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(54) Title: MODIFIED INTERFERON BETA WITH REDUCED IMMUNOGENICITY

(57) Abstract: The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of human interferon beta to result in proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*.

MODIFIED INTERFERON BETA WITH REDUCED IMMUNOGENICITY**FIELD OF THE INVENTION**

The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of human interferon and specifically human interferon beta (INF β) to result in INF β protein variants that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified INF β variants with reduced immunogenicity.

BACKGROUND OF THE INVENTION

There are many instances whereby the efficacy of a therapeutic protein is limited by an unwanted immune reaction to the therapeutic protein. Several mouse monoclonal antibodies have shown promise as therapies in a number of human disease settings but in certain cases have failed due to the induction of significant degrees of a human anti-murine antibody (HAMA) response [Schroff, R. W. et al (1985) *Cancer Res.* 45: 879-885; Shawler, D.L. et al (1985) *J. Immunol.* 135: 1530-1535]. For monoclonal antibodies, a number of techniques have been developed in attempt to reduce the HAMA response [WO 89/09622; EP 0239400; EP 0438310; WO 91/06667]. These recombinant DNA approaches have generally reduced the mouse genetic information in the final antibody construct whilst increasing the human genetic information in the final construct. Notwithstanding, the resultant "humanized" antibodies have, in several cases, still elicited an immune response in patients [Issacs J.D. (1990) *Sem. Immunol.* 2: 449, 456; Rebello, P.R. et al (1999) *Transplantation* 68: 1417-1420].

Antibodies are not the only class of polypeptide molecule administered as a therapeutic agent against which an immune response may be mounted. Even proteins of human origin and with the same amino acid sequences as occur within humans can still induce an immune response in humans. Notable examples include the therapeutic use of granulocyte-macrophage colony stimulating factor [Wadhwa, M. et al (1999) *Clin. Cancer Res.* 5: 1353-1361] and interferon alpha 2 [Russo, D. et al (1996) *Bri. J. Haem.* 94: 300-305; Stein, R. et al (1988) *New Engl. J. Med.* 318: 1409-1413] amongst others.

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A principal factor in the induction of an immune response is the presence within the protein of peptides that can stimulate the activity of T-cells via presentation on MHC class II molecules, so-called "T-cell epitopes". Such potential T-cell epitopes are commonly defined as any amino acid residue sequence with the ability to bind to MHC Class II molecules. Such T-cell epitopes can be measured to establish MHC binding. Implicitly, a "T-cell epitope" means an epitope which when bound to MHC molecules can be recognized by a T-cell receptor (TCR), and which can, at least in principle, cause the activation of these T-cells by engaging a TCR to promote a T-cell response. It is, however, usually understood that certain peptides which are found to bind to MHC Class II molecules may be retained in a protein sequence because such peptides are recognized as "self" within the organism into which the final protein is administered.

It is known, that certain of these T-cell epitope peptides can be released during the degradation of peptides, polypeptides or proteins within cells and subsequently be presented by molecules of the major histocompatibility complex (MHC) in order to trigger the activation of T-cells. For peptides presented by MHC Class II, such activation of T-cells can then give rise, for example, to an antibody response by direct stimulation of B-cells to produce such antibodies.

MHC Class II molecules are a group of highly polymorphic proteins which play a central role in helper T-cell selection and activation. The human leukocyte antigen group DR (HLA-DR) are the predominant isotype of this group of proteins and are the major focus of the present invention. However, isotypes HLA-DQ and HLA-DP perform similar functions, hence the present invention is equally applicable to these. The MHC class II DR molecule is made of an alpha and a beta chain which insert at their C-termini through the cell membrane. Each hetero-dimer possesses a ligand binding domain which binds to peptides varying between 9 and 20 amino acids in length, although the binding groove can accommodate a maximum of 11 amino acids. The ligand binding domain is comprised of amino acids 1 to 85 of the alpha chain, and amino acids 1 to 94 of the beta chain. DQ molecules have recently been shown to have an homologous structure and the DP family proteins are also expected to be very similar. In humans approximately 70 different allotypes of the DR isotype are known, for DQ there are 30 different allotypes and for DP 47 different allotypes are known. Each individual bears two to four DR alleles, two DQ and two DP alleles. The structure of a number of DR molecules has been solved and such structures point to an open-ended peptide binding groove with a

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number of hydrophobic pockets which engage hydrophobic residues (pocket residues) of the peptide [Brown et al *Nature* (1993) 364: 33; Stern et al (1994) *Nature* 368: 215].

Polymorphism identifying the different allotypes of class II molecule contributes to a wide diversity of different binding surfaces for peptides within the peptide binding groove and at the population level ensures maximal flexibility with regard to the ability to recognize foreign proteins and mount an immune response to pathogenic organisms.

There is a considerable amount of polymorphism within the ligand binding domain with distinct "families" within different geographical populations and ethnic groups. This polymorphism affects the binding characteristics of the peptide binding domain, thus different "families" of DR molecules will have specificities for peptides with different sequence properties, although there may be some overlap. This specificity determines recognition of Th-cell epitopes (Class II T-cell response) which are ultimately responsible for driving the antibody response to B-cell epitopes present on the same protein from which the Th-cell epitope is derived. Thus, the immune response to a protein in an individual is heavily influenced by T-cell epitope recognition which is a function of the peptide binding specificity of that individual's HLA-DR allotype. Therefore, in order to identify T-cell epitopes within a protein or peptide in the context of a global population, it is desirable to consider the binding properties of as diverse a set of HLA-DR allotypes as possible, thus covering as high a percentage of the world population as possible.

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An immune response to a therapeutic protein such as $\text{INF}\beta$ proceeds via the MHC class II peptide presentation pathway. Here exogenous proteins are engulfed and processed for presentation in association with MHC class II molecules of the DR, DQ or DP type. MHC Class II molecules are expressed by professional antigen presenting cells (APCs), such as macrophages and dendritic cells amongst others. Engagement of a MHC class II peptide complex by a cognate T-cell receptor on the surface of the T-cell, together with the cross-binding of certain other co-receptors such as the CD4 molecule, can induce an activated state within the T-cell. Activation leads to the release of cytokines further activating other lymphocytes such as B cells to produce antibodies or activating T killer cells as a full cellular immune response.

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The ability of a peptide to bind a given MHC class II molecule for presentation on the surface of an APC is dependent on a number of factors most notably its primary sequence. This will

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influence both its propensity for proteolytic cleavage and also its affinity for binding within the peptide binding cleft of the MHC class II molecule. The MHC class II / peptide complex on the APC surface presents a binding face to a particular T-cell receptor (TCR) able to recognize determinants provided both by exposed residues of the peptide and the MHC class II molecule.

In the art there are procedures for identifying synthetic peptides able to bind MHC class II molecules (e.g. WO98/52976 and WO00/34317). Such peptides may not function as T-cell epitopes in all situations, particularly, *in vivo* due to the processing pathways or other phenomena. T-cell epitope identification is the first step to epitope elimination. The identification and removal of potential T-cell epitopes from proteins has been previously disclosed. In the art methods have been provided to enable the detection of T-cell epitopes usually by computational means scanning for recognized sequence motifs in experimentally determined T-cell epitopes or alternatively using computational techniques to predict MHC class II-binding peptides and in particular DR-binding peptides.

WO98/52976 and WO00/34317 teach computational threading approaches to identifying polypeptide sequences with the potential to bind a sub-set of human MHC class II DR allotypes. In these teachings, predicted T-cell epitopes are removed by the use of judicious amino acid substitution within the primary sequence of the therapeutic antibody or non-antibody protein of both non-human and human derivation.

Other techniques exploiting soluble complexes of recombinant MHC molecules in combination with synthetic peptides and able to bind to T-cell clones from peripheral blood samples from human or experimental animal subjects have been used in the art [Kern, F. et al (1998) *Nature Medicine* 4:975-978; Kwok, W.W. et al (2001) *TRENDS in Immunol.* 22:583-588]. These and other schemes including for example the use of whole INF β proteins or INF β derived synthetic peptides or variant molecules thereof which are screened for molecules with altered ability to bind or stimulate T-cells may also be exploited in an epitope identification strategy.

As depicted above and as consequence thereof, it would be desirable to identify and to remove or at least to reduce T-cell epitopes from a given in principal therapeutically valuable but originally immunogenic peptide, polypeptide or protein.

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One of these therapeutically valuable molecules is INF β . The molecule is a single chain glycoprotein of 166 amino acid residues with important biological and immunological activity. The protein has significant therapeutic potential in man as an anti-viral, anti-proliferative and immunomodulating agent. There are a number of commercial sources of recombinant INF β and these include AVONEX[®]; manufactured by Biogen, Inc. (Cambridge, MA, USA); Rebif[®] manufactured by Serono International (Geneva, Switzerland) and Betaseron[®] produced by the Chiron Corporation (Emeryville, CA, USA). The amino acid sequences of AVONEX[®] and Rebif[®] are identical to that of natural human INF β and both products are glycosylated. By contrast, Betaseron[®] is produced from an *E.coli* expression host and is a mutated form of INF β where cysteine 17 has been mutated to a serine residue. It is a 165 amino acid non-glycosylated protein with a molecular weight of 18500.

The mature human INF β protein is single polypeptide of 166 amino acids with a molecular weight of 22500 and is produced by various cell types including fibroblasts and macrophages. The amino acid sequence of human INF β (depicted as one-letter code) is as follows:

MSYNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDP EEIKQLQQFQKEDAALTIYEMLQNIFAI
FRQDSSSTGWN ETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHLK RYYGRILHYLKAKEY
SHCAWTIVRVEILRN FYFINRLTG YLRN

Others have provided INF β molecules, including modified IFN β such as the mutated and aglycosylated form comprising Betaseron[®] and the series of alanine scanning mutants described by Runkel et al [Runkel, L. Et al (2000) *Biochemistry* 39: 2538-2551]. Other examples include those disclosed in US,4,588,585 and US,6,127,332 but none of these teachings recognise the importance of T cell epitopes to the immunogenic properties of the protein nor have been conceived to directly influence said properties in a specific and controlled way according to the scheme of the present invention.

However, there is a continued need for INF β analogues with enhanced properties. Desired enhancements include alternative schemes and modalities for the expression and purification of the said therapeutic, but also and especially, improvements in the biological properties of the protein. There is a particular need for enhancement of the *in vivo* characteristics when

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administered to the human subject. In this regard, it is highly desired to provide INF β with reduced or absent potential to induce an immune response in the human subject.

5 SUMMARY AND DESCRIPTION OF THE INVENTION

The present invention provides for modified forms of human interferon beta 1a, herein called "INF β ", in which the immune characteristic is modified by means of reduced or removed numbers of potential T-cell epitopes.

- 10 The invention discloses sequences identified within the INF β primary sequence that are potential T-cell epitopes by virtue of MHC class II binding potential. This disclosure specifically pertains the human INF β protein being 166 amino acid residues.

The invention discloses also specific positions within the primary sequence of the molecule which according to the invention are to be altered by specific amino acid substitution, addition
15 or deletion whilst retaining to a maximum degree the biological activity of the protein. In cases in which the loss of immunogenicity can be achieved only by a simultaneous loss of biological activity it is possible to restore said activity by further alterations within the amino acid sequence of the protein.

The invention furthermore discloses methods to produce such modified molecules, and above
20 all methods to identify said T-cell epitopes which require alteration in order to reduce or remove immunogenic sites.

The protein according to this invention would expect to display an increased circulation time within the human subject and would be of particular benefit in chronic or recurring disease settings such as is the case for a number of indications for INF β . The present invention
25 provides for modified forms of INF β proteins that are expected to display enhanced properties *in vivo*. The present invention discloses the major regions of the INF β primary sequence that are immunogenic in man and provides modification to the said sequences to eliminate or reduce the immunogenic effectiveness of these sites. In one embodiment, synthetic peptides comprising the said immunogenic regions can be provided in pharmaceutical composition for
30 the purpose of promoting a tolerogenic response to the whole molecule. In a further embodiment, the modified INF β molecules of the present invention can be used in pharmaceutical compositions.

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In summary the invention relates to the following issues:

- a modified molecule having the biological activity of $\text{INF}\beta$ and being substantially non-immunogenic or less immunogenic than any non-modified molecule having the same biological activity when used *in vivo*;
- 5 • an accordingly specified molecule, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule;
- an accordingly specified molecule, wherein said loss of immunogenicity is achieved by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule;
- an accordingly specified molecule, wherein one T-cell epitope is removed;
- 10 • an accordingly specified molecule, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II;
- an accordingly specified molecule, wherein said peptide sequences are selected from the group as depicted in FIGURE 1;
- 15 • an accordingly specified molecule, wherein 1 – 9 amino acid residues, preferably one amino acid residue in any of the originally present T-cell epitopes are altered;
- an accordingly specified molecule, wherein the alteration of the amino acid residues is substitution, addition or deletion of originally present amino acid(s) residue(s) by other amino acid residue(s) at specific position(s);
- 20 • an accordingly specified molecule, wherein one or more of the amino acid residue substitutions are carried out as indicated in FIGURE 2;
- an accordingly specified molecule, wherein (additionally) one or more of the amino acid residue substitutions are carried out as indicated in FIGURE 3 for the reduction in the number of MHC allotypes able to bind peptides derived from said molecule;
- 25 • an accordingly specified molecule, wherein one or more amino acid residue substitutions are carried out as indicted in FIGURE 4.
- an accordingly specified molecule, wherein, if necessary, additionally further alteration usually by substitution, addition or deletion of specific amino acid(s) is conducted to restore biological activity of said molecule;
- 30 • an accordingly specified molecule wherein alteration is conducted at one or more residues from the string of contiguous residues of sequence (a) QFQKEDAALTIYEMLQNIFAIFRQ

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(R1) and / or of sequence; (b) RYYGRILHYLKAKEYSHCAWT (R2) wherein said sequences are derived from the INF β wild-type sequence;

- a peptide molecule comprising 13–15 consecutive residues from any of sequences (a) or (b) above;
- 5 • a peptide molecule comprising at least 9 consecutive residues from any of the sequences (a) or (b) above;
- a peptide molecule of above sharing greater than 90% amino acid identity with any of the peptide sequences derived from (a) or (b) above;
- a peptide molecule of above sharing greater than 80% amino acid identity with any of the peptide sequences derived from (a) or (b) above;
- 10 • peptide sequences as above able to bind MHC class II;
- an accordingly specified INF β molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (a) above;
- 15 • an accordingly specified INF β molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (b) above;
- an accordingly specified INF β molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequences (a) or (b) above;
- 20 • a modified human interferon beta (INF β) having reduced immunogenicity consisting of the following sequence:
MSYNLLGFLQRSSNFQX⁰QKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQX¹QKEDAAAX²TX³X⁴EX⁵X⁶QNX⁷X⁸AX⁹X¹⁰RQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHLKRY
25 GRILHYLKAKEYSHCAWTIVRVEILRNIFYFINRLTGYLRLN
wherein X⁰ is C, S; X¹ is F, A; X² is L, A; X³ is I, A; X⁴ is Y, N; X⁵ is M, A; X⁶ is L, A; X⁷ is I, T; X⁸ is F, H; X⁹ is I, A and X¹⁰ is F, A;
whereby simultaneously X¹ = F, X² = L, X³ = I, X⁴ = Y, X⁵ = M, X⁶ = L, X⁷ = I, X⁸ = F, X⁹ = I and X¹⁰ = F are excluded (these exclusions describe the known immunogenetically non-
30 modified IFNb variants);
- a modified human interferon beta (INF β) having reduced immunogenicity consisting of the following sequence:

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MSYNLLGFLQRSSNFQX⁰QKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAALTIYEMLQNI
FAIFRQDSSSTGWNENITIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHLKRX¹X²GRX³X⁴
HX⁵X⁶KAKEX⁷SHCAWTIVRVEILRNIFYFINRLTGYLRLN,

wherein X⁰ is C, S; X¹ is Y, A; X² is Y, A; X³ is I, A; X⁴ is L, A; X⁵ is Y, S; X⁶ is L, A and
5 X⁷ is Y, H, A; whereby simultaneously X¹ = Y, X² = Y, X³ = I, X⁴ = L, X⁵ = Y, X⁶ = L and
X⁷ = Y are excluded. (these exclusions describe the known immunogenetically non-modified
IFN β variants);

- an IFN beta molecule consisting of 9 – 15 consecutive amino acid residues, having a
potential MHC class II binding activity and created from the primary sequence of non-
10 modified IFN β , whereby said molecule has a stimulation index of at least at least 1.8,
preferably 1.8 – 2, more preferably > 2, in a biological assay of cellular proliferation
wherein said index is taken as the value of cellular proliferation scored following
stimulation by a peptide and divided by the value of cellular proliferation scored in control
cells not in receipt peptide and wherein cellular proliferation is measured by any suitable
15 means;
- a pharmaceutical composition comprising any of the peptides or modified peptides of above
having the activity of binding to MHC class II
- a DNA sequence or molecule which codes for any of said specified modified molecules as
defined above and below;
- 20 • a pharmaceutical composition comprising a modified molecule having the biological
activity of IFN β
- a pharmaceutical composition as defined above and / or in the claims, optionally together
with a pharmaceutically acceptable carrier, diluent or excipient;
- a method for manufacturing a modified molecule having the biological activity of IFN β as
25 defined in any of the claims of the above-cited claims comprising the following steps: (i)
determining the amino acid sequence of the polypeptide or part thereof; (ii) identifying one
or more potential T-cell epitopes within the amino acid sequence of the protein by any
method including determination of the binding of the peptides to MHC molecules using *in*
vitro or *in silico* techniques or biological assays; (iii) designing new sequence variants with
30 one or more amino acids within the identified potential T-cell epitopes modified in such a
way to substantially reduce or eliminate the activity of the T-cell epitope as determined by
the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or
biological assays; (iv) constructing such sequence variants by recombinant DNA techniques

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and testing said variants in order to identify one or more variants with desirable properties; and (v) optionally repeating steps (ii) – (iv);

- an accordingly specified method, wherein step (iii) is carried out by substitution, addition or deletion of 1 – 9 amino acid residues in any of the originally present T-cell epitopes;
- 5 • an accordingly specified method, wherein the alteration is made with reference to an homologous protein sequence and / or *in silico* modeling techniques;
- an accordingly specified method, wherein step (ii) of above is carried out by the following steps: (a) selecting a region of the peptide having a known amino acid residue sequence; (b) sequentially sampling overlapping amino acid residue segments of predetermined uniform
10 size and constituted by at least three amino acid residues from the selected region; (c) calculating MHC Class II molecule binding score for each said sampled segment by summing assigned values for each hydrophobic amino acid residue side chain present in said sampled amino acid residue segment; and (d) identifying at least one of said segments suitable for modification, based on the calculated MHC Class II molecule binding score for
15 that segment, to change overall MHC Class II binding score for the peptide without substantially reducing therapeutic utility of the peptide; step (c) is preferably carried out by using a Böhm scoring function modified to include 12-6 van der Waal's ligand-protein energy repulsive term and ligand conformational energy term by (1) providing a first data base of MHC Class II molecule models; (2) providing a second data base of allowed
20 peptide backbones for said MHC Class II molecule models; (3) selecting a model from said first data base; (4) selecting an allowed peptide backbone from said second data base; (5) identifying amino acid residue side chains present in each sampled segment; (6) determining the binding affinity value for all side chains present in each sampled segment; and repeating steps (1) through (5) for each said model and each said backbone;
- 25 • a 13mer T-cell epitope peptide having a potential MHC class II binding activity and created from non-modified INF β , selected from the group as depicted in FIGURE 1 and its use for the manufacture of INF β having substantially no or less immunogenicity than any non-modified molecule with the same biological activity when used *in vivo*;
- a peptide sequence consisting of at least 9 consecutive amino acid residues of a 13mer T-
30 cell epitope peptide as specified above and its use for the manufacture of INF β having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of a human interferon β when used *in vivo*;

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- a 13mer T-cell epitope peptide having a potential MHC class II binding activity and created from non-modified INF β , selected from any of the group of sequences (a) or (b) as defined above and its use for the manufacture of INF β having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of a human interferon β when used *in vivo*;
- a peptide sequence consisting of at least 9 consecutive amino acid residues of a 13mer T-cell epitope peptide as derived from any of the sequences (a) or (b) as specified above, and its use for the manufacture of INF β having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of a human interferon β when used *in vivo*.

The term "T-cell epitope" means according to the understanding of this invention an amino acid sequence which is able to bind MHC class II, able to stimulate T-cells and / or also to bind (without necessarily measurably activating) T-cells in complex with MHC class II.

The term "peptide" as used herein and in the appended claims, is a compound that includes two or more amino acids. The amino acids are linked together by a peptide bond (defined herein below). There are 20 different naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have the L-configuration. Synthetic peptides can be prepared employing conventional synthetic methods, utilizing L-amino acids, D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid units. Short peptides, e.g., having less than ten amino acid units, are sometimes referred to as "oligopeptides". Other peptides contain a large number of amino acid residues, e.g. up to 100 or more, and are referred to as "polypeptides". By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acids, whereas a "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any reference to a "polypeptide" also includes an oligopeptide. Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acids forms different polypeptides or proteins. The number of polypeptides—and hence the number of different proteins—that can be formed is practically unlimited.

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"Alpha carbon (C α)" is the carbon atom of the carbon-hydrogen (CH) component that is in the peptide chain. A "side chain" is a pendant group to C α that can comprise a simple or complex group or moiety, having physical dimensions that can vary significantly compared to the dimensions of the peptide.

- 5 The invention may be applied to any INF β species of molecule with substantially the same primary amino acid sequences as those disclosed herein and would include therefore INF β molecules derived by genetic engineering means or other processes and may contain more or less than 166 amino acid residues.

INF β proteins such as identified from other mammalian sources have in common many of the
10 peptide sequences of the present disclosure and have in common many peptide sequences with substantially the same sequence as those of the disclosed listing. Such protein sequences equally therefore fall under the scope of the present invention.

The invention is conceived to overcome the practical reality that soluble proteins introduced
15 into autologous organisms can trigger an immune response resulting in development of host antibodies that bind to the soluble protein. A prominent example of this phenomenon amongst others, is the clinical use of interferon alpha 2 (INF α 2). A significant proportion of human patients treated with INF α 2 make antibodies despite the fact that this protein is produced endogenously [Russo, D. et al (1996) *ibid*; Stein, R. et al (1988) *ibid*]. It is known that the
20 clinical use of INF β has also resulted in the development of immune responses to the INF β despite the fact that a molecule of at least identical primary structure is produced endogenously in man [Kivisakk, P. et al (2000) *Eur. J. Neurol.* 7: 27-34; Myhr, K.M. et al (2000) *Neurology* 55: 1569-1572]. The present invention seeks to address this by providing INF β proteins with altered propensity to elicit an immune response on administration to the
25 human host. According to the methods described herein, the inventors have discovered and now disclose the regions of the INF β molecule comprising the critical T-cell epitopes driving the immune responses to this autologous protein.

The general method of the present invention leading to the modified INF β comprises the
30 following steps:

- (a) determining the amino acid sequence of the polypeptide or part thereof;

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- (b) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays;
- (c) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays. Such sequence variants are created in such a way to avoid creation of new potential T-cell epitopes by the sequence variations unless such new potential T-cell epitopes are, in turn, modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope; and
- (d) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties according to well known recombinant techniques.
- 15 The identification of potential T-cell epitopes according to step (b) can be carried out according to methods describes previously in the prior art. Suitable methods are disclosed in WO 98/59244; WO 98/52976; WO 00/34317 and may preferably be used to identify binding propensity of INF β -derived peptides to an MHC class II molecule.
- 20 Another very efficacious method for identifying T-cell epitopes by calculation is described in the EXAMPLE 1 which is a preferred embodiment according to this invention.

In practice a number of variant INF β proteins will be produced and tested for the desired immune and functional characteristic. The variant proteins will most preferably be produced by recombinant DNA techniques although other procedures including chemical synthesis of INF β fragments may be contemplated. Chemical synthesis is for example particularly preferred for the production of short INF β fragments such as the R1 or R2 sequence elements disclosed herein and which comprise a particular embodiment of the present invention.

- 30 The results of an analysis according to step (b) of the above scheme and pertaining to the human INF β protein sequence of 166 amino acid residues is presented in FIGURE 1. The results of a design and constructs according to step (c) and (d) of the above scheme and pertaining to the modified molecule of this invention is presented in FIGURES 2 and 3.

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The invention relates to INF β analogues in which substitutions of at least one amino acid residue have been made at positions resulting in a substantial reduction in activity of or elimination of one or more potential T-cell epitopes from the protein. One or more amino acid substitutions at particular points within any of the potential MHC class II ligands identified in

5 FIGURE 1 may result in a INF β molecule with a reduced immunogenic potential when administered as a therapeutic to the human host.

It is most preferred to provide an INF β molecule in which amino acid modification (e.g. a substitution) is conducted within the most immunogenic regions of the parent molecule. The

10 inventors herein have discovered that the most immunogenic regions of the INF β molecule in man are confined to two regions R1 and R2 comprising respectively amino acid sequences; QFQKEDAALTIYEMLQNIFAIFRQ and RYYGRILHYLKAKEYSHCAWT. The major preferred embodiments of the present invention comprise INF β molecules for which the MHC class II ligands of FIGURE 1 and which align either in their entirety or to a minimum of 9 amino acid

15 residues with any of the above sequence elements R1 or R2 are altered such as to eliminate binding or otherwise reduce the numbers of MHC allotypes to which the peptide can bind.

The preferred embodiments of the invention include the specific substitutions of FIGURE 4. It is particularly preferred to provide modified INF β molecules containing combinations of

20 substitutions from FIGURE 4. Combinations which comprise multiple (greater than 1) modification both within each of the immunogenic regions R1 and R2, and combinations comprising multiple modifications to both R1 and R2 within the same molecule are especially preferred although such preference is not intended to limit the combinations of substitution which are considered desirable.

25

For the elimination of T-cell epitopes, amino acid substitutions are preferably made at appropriate points within the peptide sequence predicted to achieve substantial reduction or elimination of the activity of the T-cell epitope. In practice an appropriate point will preferably equate to an amino acid residue binding within one of the pockets provided within

30 the MHC class II binding groove.

It is most preferred to alter binding within the first pocket of the cleft at the so-called P1 or P1 anchor position of the peptide. The quality of binding interaction between the P1 anchor

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residue of the peptide and the first pocket of the MHC class II binding groove is recognized as being a major determinant of overall binding affinity for the whole peptide. An appropriate substitution at this position of the peptide will be for a residue less readily accommodated within the pocket, for example, substitution to a more hydrophilic residue. Amino acid
5 residues in the peptide at positions equating to binding within other pocket regions within the MHC binding cleft are also considered and fall under the scope of the present.

It is understood that single amino acid substitutions within a given potential T-cell epitope are the most preferred route by which the epitope may be eliminated. Combinations of
10 substitution within a single epitope may be contemplated and for example can be particularly appropriate where individually defined epitopes are in overlap with each other. Moreover, amino acid substitutions either singly within a given epitope or in combination within a single epitope may be made at positions not equating to the "pocket residues" with respect to the MHC class II binding groove, but at any point within the peptide sequence. Substitutions may
15 be made with reference to an homologues structure or structural method produced using *in silico* techniques known in the art and may be based on known structural features of the molecule according to this invention. All such substitutions fall within the scope of the present invention.

20 Amino acid substitutions other than within the peptides identified above may be contemplated particularly when made in combination with substitution(s) made within a listed peptide. For example a change may be contemplated to restore structure or biological activity of the variant molecule. Such compensatory changes and changes to include deletion or addition of particular amino acid residues from the INF β polypeptide resulting in a variant with desired
25 activity and in combination with changes in any of the disclosed peptides fall under the scope of the present.

In as far as this invention relates to modified INF β , compositions containing such modified INF β proteins or fragments of modified INF β proteins and related compositions should be considered within the scope of the invention. In another aspect, the present invention relates
30 to nucleic acids encoding modified INF β entities. In a further aspect the present invention relates to methods for therapeutic treatment of humans using the modified INF β proteins. In a further aspect still, the invention relates to methods for therapeutic treatment using

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pharmaceutical preparations comprising peptide or derivative molecules with sequence identity or part identity with the sequences herein disclosed as R1 or R2.

The invention will now be illustrated, but not limited, by the following examples. The
5 examples refer to the following drawings:

Figure 1 provides a table of peptide sequences in human INF β with potential human MHC class II binding activity. Peptides are 13mers, amino acids are identified using single letter code

10 Figure 2 provides a table detailing amino acid substitutions leading to the elimination of T-cell epitopes of human INF β . WT = wild type residue.

Figure 3 provides a table detailing additional substitutions leading to the removal of a potential T-cell epitope for 1 or more MHC allotypes.

Figure 4 provides a table of preferred substitutions in human INF β . WT = wild type residue;
15 # = position; MUT = desired residue. The table indicates the epitope region (R1 or R2) in which each substitution is located.

Figure 5 provides a table of the INF β 15-mer peptide sequences analysed using the naïve human *in vitro* T-cell assay of EXAMPLE 2. The peptide ID# and position of the N-terminal peptide residue within the INF β sequence is indicated.

20 Figure 6 shows cumulative stimulation indexes from 6 individuals that respond to stimulation with INF β peptides. Panel 6a shows results following stimulation using peptides at 1 μ M concentration. Panel 6b shows results following stimulation using peptides at 10 μ M concentration. Six donors from 20 screened responded to stimulation with one or more peptides from the INF β sequence. Responses to individual peptides are grouped into two
25 distinct regions R1 and R2. Control peptides C32 (DRB1-restricted) and C49 (DP-restricted) are included for comparison. Cross-hatching within each bar indicates the contribution from individual donors. SI = stimulation index.

Figure 7 shows the donor specific stimulation responses to the INF β synthetic peptides.

Panels 7a- 7f show individual donor responses to peptides at 1 μ M (light bars) and 10 μ M (dark
30 bars) final peptide concentration. Data from control peptides C32 (DRB1-restricted) and C49 (DP-restricted) are included in each panel for comparison. Threshold for positive stimulation index = 2.

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Figure 8 shows the immunogenic regions within INF β and details the peptide sequences from these regions able to stimulate naïve human T-cells.

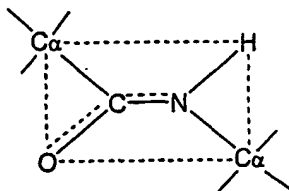
Figure 9 provides a table indicating INF β peptides capable of promoting proliferation of naïve human T-cells *in vitro*. For two of the donors, responses are recorded to multiple overlapping peptides from either epitope region R1 or R2. Responses to individual synthetic peptides mapping to epitope regions R1 or R2 are scored from six donors.

Figure 10 provides representative data of the anti-proliferative effect of two modified INF β molecules. Assays were conducted according to the methods of EXAMPLE 4. In each of panels a) and b), antiproliferative effects of control treatments are recorded. Controls comprise non-modified INF β -Fc fusion = WT-FcINF β ; a standard INF β preparation = R&D IFN β and media containing no INF = Media Con. Panel a) shows data for Leu 57 Ala (INF β -BIOV7) modified INF β . Panel b) shows data for the Phe 67 His (INF β -BIOV12) modified INF β .

EXAMPLE 1

There are a number of factors that play important roles in determining the total structure of a protein or polypeptide. First, the peptide bond, i.e., that bond which joins the amino acids in the chain together, is a covalent bond. This bond is planar in structure, essentially a substituted amide. An "amide" is any of a group of organic compounds containing the grouping -CONH-.

The planar peptide bond linking C α of adjacent amino acids may be represented as depicted below:



Because the O=C and the C-N atoms lie in a relatively rigid plane, free rotation does not occur about these axes. Hence, a plane schematically depicted by the interrupted line is sometimes referred to as an "amide" or "peptide plane" plane wherein lie the oxygen (O), carbon (C),

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nitrogen (N), and hydrogen (H) atoms of the peptide backbone. At opposite corners of this amide plane are located the C α atoms. Since there is substantially no rotation about the O=C and C-N atoms in the peptide or amide plane, a polypeptide chain thus comprises a series of planar peptide linkages joining the C α atoms.

5 A second factor that plays an important role in defining the total structure or conformation of a polypeptide or protein is the angle of rotation of each amide plane about the common C α linkage. The terms "angle of rotation" and "torsion angle" are hereinafter regarded as equivalent terms. Assuming that the O, C, N, and H atoms remain in the amide plane (which is usually a valid assumption, although there may be some slight deviations from planarity of
10 these atoms for some conformations), these angles of rotation define the N and R polypeptide's backbone conformation, i.e., the structure as it exists between adjacent residues. These two angles are known as ϕ and ψ . A set of the angles ϕ_i, ψ_i , where the subscript i represents a particular residue of a polypeptide chain, thus effectively defines the polypeptide secondary structure. The conventions used in defining the ϕ, ψ angles, i.e., the reference
15 points at which the amide planes form a zero degree angle, and the definition of which angle is ϕ , and which angle is ψ , for a given polypeptide, are defined in the literature. See, e.g., Ramachandran et al. *Adv. Prot. Chem.* 23:283-437 (1968), at pages 285-94, which pages are incorporated herein by reference.

20 The present method can be applied to any protein, and is based in part upon the discovery that in humans the primary Pocket 1 anchor position of MHC Class II molecule binding grooves has a well designed specificity for particular amino acid side chains. The specificity of this pocket is determined by the identity of the amino acid at position 86 of the beta chain of the MHC Class II molecule. This site is located at the bottom of Pocket 1 and determines the size
25 of the side chain that can be accommodated by this pocket. Marshall, K.W., *J. Immunol.*, 152:4946-4956 (1994). If this residue is a glycine, then all hydrophobic aliphatic and aromatic amino acids (hydrophobic aliphatics being: valine, leucine, isoleucine, methionine and aromatics being: phenylalanine, tyrosine and tryptophan) can be accommodated in the pocket, a preference being for the aromatic side chains. If this pocket residue is a valine, then the side
30 chain of this amino acid protrudes into the pocket and restricts the size of peptide side chains that can be accommodated such that only hydrophobic aliphatic side chains can be accommodated. Therefore, in an amino acid residue sequence, wherever an amino acid with a hydrophobic aliphatic or aromatic side chain is found, there is the potential for a MHC Class II

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restricted T-cell epitope to be present. If the side-chain is hydrophobic aliphatic, however, it is approximately twice as likely to be associated with a T-cell epitope than an aromatic side chain (assuming an approximately even distribution of Pocket 1 types throughout the global population).

5 A computational method embodying the present invention profiles the likelihood of peptide regions to contain T-cell epitopes as follows:

- (1) The primary sequence of a peptide segment of predetermined length is scanned, and all hydrophobic aliphatic and aromatic side chains present are identified. (2) The hydrophobic aliphatic side chains are assigned a value greater than that for the aromatic side chains; preferably about twice the value assigned to the aromatic side chains, e.g., a value of 2 for a hydrophobic aliphatic side chain and a value of 1 for an aromatic side chain. (3) The values determined to be present are summed for each overlapping amino acid residue segment (window) of predetermined uniform length within the peptide, and the total value for a particular segment (window) is assigned to a single amino acid residue at an intermediate position of the segment (window), preferably to a residue at about the midpoint of the sampled segment (window). This procedure is repeated for each sampled overlapping amino acid residue segment (window). Thus, each amino acid residue of the peptide is assigned a value that relates to the likelihood of a T-cell epitope being present in that particular segment (window). (4) The values calculated and assigned as described in Step 3, above, can be plotted against the amino acid coordinates of the entire amino acid residue sequence being assessed. (5) All portions of the sequence which have a score of a predetermined value, e.g., a value of 1, are deemed likely to contain a T-cell epitope and can be modified, if desired.

This particular aspect of the present invention provides a general method by which the regions of peptides likely to contain T-cell epitopes can be described. Modifications to the peptide in these regions have the potential to modify the MHC Class II binding characteristics.

According to another aspect of the present invention, T-cell epitopes can be predicted with greater accuracy by the use of a more sophisticated computational method which takes into account the interactions of peptides with models of MHC Class II alleles.

The computational prediction of T-cell epitopes present within a peptide according to this particular aspect contemplates the construction of models of at least 42 MHC Class II alleles based upon the structures of all known MHC Class II molecules and a method for the use of these models in the computational identification of T-cell epitopes, the construction of libraries of peptide backbones for each model in order to allow for the known variability in

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relative peptide backbone alpha carbon (C α) positions, the construction of libraries of amino-acid side chain conformations for each backbone dock with each model for each of the 20 amino-acid alternatives at positions critical for the interaction between peptide and MHC Class II molecule, and the use of these libraries of backbones and side-chain conformations in conjunction with a scoring function to select the optimum backbone and side-chain conformation for a particular peptide docked with a particular MHC Class II molecule and the derivation of a binding score from this interaction.

Models of MHC Class II molecules can be derived via homology modeling from a number of similar structures found in the Brookhaven Protein Data Bank ("PDB"). These may be made by the use of semi-automatic homology modeling software (Modeller, Sali A. & Blundell TL., 1993. *J. Mol Biol* 234:779-815) which incorporates a simulated annealing function, in conjunction with the CHARMM force-field for energy minimisation (available from Molecular Simulations Inc., San Diego, Ca.). Alternative modeling methods can be utilized as well.

The present method differs significantly from other computational methods which use libraries of experimentally derived binding data of each amino-acid alternative at each position in the binding groove for a small set of MHC Class II molecules (Marshall, K.W., *et al.*, *Biomed. Pept. Proteins Nucleic Acids*, 1(3):157-162) (1995) or yet other computational methods which use similar experimental binding data in order to define the binding characteristics of particular types of binding pockets within the groove, again using a relatively small subset of MHC Class II molecules, and then 'mixing and matching' pocket types from this pocket library to artificially create further 'virtual' MHC Class II molecules (Sturniolo T., *et al.*, *Nat. Biotech*, 17(6): 555-561 (1999). Both prior methods suffer the major disadvantage that, due to the complexity of the assays and the need to synthesize large numbers of peptide variants, only a small number of MHC Class II molecules can be experimentally scanned. Therefore the first prior method can only make predictions for a small number of MHC Class II molecules. The second prior method also makes the assumption that a pocket lined with similar amino-acids in one molecule will have the same binding characteristics when in the context of a different Class II allele and suffers further disadvantages in that only those MHC Class II molecules can be 'virtually' created which contain pockets contained within the pocket library. Using the modeling approach described herein, the structure of any number and type of MHC Class II molecules can be deduced, therefore alleles can be specifically selected to be representative of the global population. In addition, the number of MHC Class II molecules scanned can be

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increased by making further models further than having to generate additional data via complex experimentation.

The use of a backbone library allows for variation in the positions of the C α atoms of the various peptides being scanned when docked with particular MHC Class II molecules. This is again in contrast to the alternative prior computational methods described above which rely on the use of simplified peptide backbones for scanning amino-acid binding in particular pockets. These simplified backbones are not likely to be representative of backbone conformations found in 'real' peptides leading to inaccuracies in prediction of peptide binding. The present backbone library is created by superposing the backbones of all peptides bound to MHC Class II molecules found within the Protein Data Bank and noting the root mean square (RMS) deviation between the C α atoms of each of the eleven amino-acids located within the binding groove. While this library can be derived from a small number of suitable available mouse and human structures (currently 13), in order to allow for the possibility of even greater variability, the RMS figure for each C"- α position is increased by 50%. The average C α position of each amino-acid is then determined and a sphere drawn around this point whose radius equals the RMS deviation at that position plus 50%. This sphere represents all allowed C α positions.

Working from the C α with the least RMS deviation (that of the amino-acid in Pocket 1 as mentioned above, equivalent to Position 2 of the 11 residues in the binding groove), the sphere is three-dimensionally gridded, and each vertex within the grid is then used as a possible location for a C α of that amino-acid. The subsequent amide plane, corresponding to the peptide bond to the subsequent amino-acid is grafted onto each of these C α s and the ϕ and ψ angles are rotated step-wise at set intervals in order to position the subsequent C α . If the subsequent C α falls within the 'sphere of allowed positions' for this C α than the orientation of the dipeptide is accepted, whereas if it falls outside the sphere then the dipeptide is rejected. This process is then repeated for each of the subsequent C α positions, such that the peptide grows from the Pocket 1 C α 'seed', until all nine subsequent C α s have been positioned from all possible permutations of the preceding C α s. The process is then repeated once more for the single C α preceding pocket 1 to create a library of backbone C α positions located within the binding groove.

The number of backbones generated is dependent upon several factors: The size of the 'spheres of allowed positions'; the fineness of the gridding of the 'primary sphere' at the

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Pocket 1 position; the fineness of the step-wise rotation of the ϕ and ψ angles used to position subsequent C α s. Using this process, a large library of backbones can be created. The larger the backbone library, the more likely it will be that the optimum fit will be found for a particular peptide within the binding groove of an MHC Class II molecule. Inasmuch as all
5 backbones will not be suitable for docking with all the models of MHC Class II molecules due to clashes with amino-acids of the binding domains, for each allele a subset of the library is created comprising backbones which can be accommodated by that allele.

The use of the backbone library, in conjunction with the models of MHC Class II molecules creates an exhaustive database consisting of allowed side chain conformations for each amino-
10 acid in each position of the binding groove for each MHC Class II molecule docked with each allowed backbone. This data set is generated using a simple steric overlap function where a MHC Class II molecule is docked with a backbone and an amino-acid side chain is grafted onto the backbone at the desired position. Each of the rotatable bonds of the side chain is rotated step-wise at set intervals and the resultant positions of the atoms dependent upon that
15 bond noted. The interaction of the atom with atoms of side-chains of the binding groove is noted and positions are either accepted or rejected according to the following criteria: The sum total of the overlap of all atoms so far positioned must not exceed a pre-determined value. Thus the stringency of the conformational search is a function of the interval used in the step-wise rotation of the bond and the pre-determined limit for the total overlap. This latter value
20 can be small if it is known that a particular pocket is rigid, however the stringency can be relaxed if the positions of pocket side-chains are known to be relatively flexible. Thus allowances can be made to imitate variations in flexibility within pockets of the binding groove. This conformational search is then repeated for every amino-acid at every position of each backbone when docked with each of the MHC Class II molecules to create the exhaustive
25 database of side-chain conformations.

A suitable mathematical expression is used to estimate the energy of binding between models of MHC Class II molecules in conjunction with peptide ligand conformations which have to be empirically derived by scanning the large database of backbone/side-chain conformations described above. Thus a protein is scanned for potential T-cell epitopes by subjecting each
30 possible peptide of length varying between 9 and 20 amino-acids (although the length is kept constant for each scan) to the following computations: An MHC Class II molecule is selected together with a peptide backbone allowed for that molecule and the side-chains corresponding to the desired peptide sequence are grafted on. Atom identity and interatomic distance data

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relating to a particular side-chain at a particular position on the backbone are collected for each allowed conformation of that amino-acid (obtained from the database described above). This is repeated for each side-chain along the backbone and peptide scores derived using a scoring function. The best score for that backbone is retained and the process repeated for each allowed backbone for the selected model. The scores from all allowed backbones are compared and the highest score is deemed to be the peptide score for the desired peptide in that MHC Class II model. This process is then repeated for each model with every possible peptide derived from the protein being scanned, and the scores for peptides versus models are displayed.

- 10 In the context of the present invention, each ligand presented for the binding affinity calculation is an amino-acid segment selected from a peptide or protein as discussed above. Thus, the ligand is a selected stretch of amino acids about 9 to 20 amino acids in length derived from a peptide, polypeptide or protein of known sequence. The terms "amino acids" and "residues" are hereinafter regarded as equivalent terms.
- 15 The ligand, in the form of the consecutive amino acids of the peptide to be examined grafted onto a backbone from the backbone library, is positioned in the binding cleft of an MHC Class II molecule from the MHC Class II molecule model library via the coordinates of the C'- α atoms of the peptide backbone and an allowed conformation for each side-chain is selected from the database of allowed conformations. The relevant atom identities and interatomic distances are also retrieved from this database and used to calculate the peptide binding score. Ligands with a high binding affinity for the MHC Class II binding pocket are flagged as candidates for site-directed mutagenesis. Amino-acid substitutions are made in the flagged ligand (and hence in the protein of interest) which is then retested using the scoring function in order to determine changes which reduce the binding affinity below a predetermined threshold value. These changes can then be incorporated into the protein of interest to remove T-cell epitopes.

Binding between the peptide ligand and the binding groove of MHC Class II molecules involves non-covalent interactions including, but not limited to: hydrogen bonds, electrostatic interactions, hydrophobic (lipophilic) interactions and Van der Waals interactions. These are included in the peptide scoring function as described in detail below.

It should be understood that a hydrogen bond is a non-covalent bond which can be formed between polar or charged groups and consists of a hydrogen atom shared by two other atoms. The hydrogen of the hydrogen donor has a positive charge where the hydrogen acceptor has a

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partial negative charge. For the purposes of peptide/protein interactions, hydrogen bond donors may be either nitrogens with hydrogen attached or hydrogens attached to oxygen or nitrogen. Hydrogen bond acceptor atoms may be oxygens not attached to hydrogen, nitrogens with no hydrogens attached and one or two connections, or sulphurs with only one connection.

5 Certain atoms, such as oxygens attached to hydrogens or imine nitrogens (e.g. C=NH) may be both hydrogen acceptors or donors. Hydrogen bond energies range from 3 to 7 Kcal/mol and are much stronger than Van der Waal's bonds, but weaker than covalent bonds. Hydrogen bonds are also highly directional and are at their strongest when the donor atom, hydrogen atom and acceptor atom are co-linear.

10 Electrostatic bonds are formed between oppositely charged ion pairs and the strength of the interaction is inversely proportional to the square of the distance between the atoms according to Coulomb's law. The optimal distance between ion pairs is about 2.8Å. In protein/peptide interactions, electrostatic bonds may be formed between arginine, histidine or lysine and aspartate or glutamate. The strength of the bond will depend upon the pKa of the ionizing

15 group and the dielectric constant of the medium although they are approximately similar in strength to hydrogen bonds.

Lipophilic interactions are favorable hydrophobic-hydrophobic contacts that occur between the protein and peptide ligand. Usually, these will occur between hydrophobic amino acid side chains of the peptide buried within the pockets of the binding groove such that they are not

20 exposed to solvent. Exposure of the hydrophobic residues to solvent is highly unfavorable since the surrounding solvent molecules are forced to hydrogen bond with each other forming cage-like clathrate structures. The resultant decrease in entropy is highly unfavorable.

Lipophilic atoms may be sulphurs which are neither polar nor hydrogen acceptors and carbon atoms which are not polar.

25 Van der Waal's bonds are non-specific forces found between atoms which are 3-4Å apart. They are weaker and less specific than hydrogen and electrostatic bonds. The distribution of electronic charge around an atom changes with time and, at any instant, the charge distribution is not symmetric. This transient asymmetry in electronic charge induces a similar asymmetry in neighboring atoms. The resultant attractive forces between atoms reaches a maximum at

30 the Van der Waal's contact distance but diminishes very rapidly at about 1Å to about 2Å. Conversely, as atoms become separated by less than the contact distance, increasingly strong repulsive forces become dominant as the outer electron clouds of the atoms overlap. Although the attractive forces are relatively weak compared to electrostatic and hydrogen bonds (about

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0.6 Kcal/mol), the repulsive forces in particular may be very important in determining whether a peptide ligand may bind successfully to a protein.

In one embodiment, the Böhm scoring function (SCORE1 approach) is used to estimate the binding constant. (Böhm, H.J., *J. Comput Aided Mol. Des.*, 8(3):243-256 (1994) which is hereby incorporated in its entirety). In another embodiment, the scoring function (SCORE2 approach) is used to estimate the binding affinities as an indicator of a ligand containing a T-cell epitope (Böhm, H.J., *J. Comput Aided Mol. Des.*, 12(4):309-323 (1998) which is hereby incorporated in its entirety). However, the Böhm scoring functions as described in the above references are used to estimate the binding affinity of a ligand to a protein where it is already known that the ligand successfully binds to the protein and the protein/ligand complex has had its structure solved, the solved structure being present in the Protein Data Bank ("PDB"). Therefore, the scoring function has been developed with the benefit of known positive binding data. In order to allow for discrimination between positive and negative binders, a repulsion term must be added to the equation. In addition, a more satisfactory estimate of binding energy is achieved by computing the lipophilic interactions in a pairwise manner rather than using the area based energy term of the above Böhm functions.

Therefore, in a preferred embodiment, the binding energy is estimated using a modified Böhm scoring function. In the modified Böhm scoring function, the binding energy between protein and ligand (ΔG_{bind}) is estimated considering the following parameters: The reduction of binding energy due to the overall loss of translational and rotational entropy of the ligand (ΔG_0); contributions from ideal hydrogen bonds (ΔG_{hb}) where at least one partner is neutral; contributions from unperturbed ionic interactions (ΔG_{ionic}); lipophilic interactions between lipophilic ligand atoms and lipophilic acceptor atoms (ΔG_{lipo}); the loss of binding energy due to the freezing of internal degrees of freedom in the ligand, i.e., the freedom of rotation about each C-C bond is reduced (ΔG_{rot}); the energy of the interaction between the protein and ligand (E_{vdw}). Consideration of these terms gives equation 1:

$$(\Delta G_{\text{bind}}) = (\Delta G_0) + (\Delta G_{\text{hb}} \times N_{\text{hb}}) + (\Delta G_{\text{ionic}} \times N_{\text{ionic}}) + (\Delta G_{\text{lipo}} \times N_{\text{lipo}}) + (\Delta G_{\text{rot}} \times N_{\text{rot}}) + (E_{\text{vdw}}).$$

Where N is the number of qualifying interactions for a specific term and, in one embodiment, ΔG_0 , ΔG_{hb} , ΔG_{ionic} , ΔG_{lipo} and ΔG_{rot} are constants which are given the values: 5.4, -4.7, -4.7, -0.17, and 1.4, respectively.

The term N_{hb} is calculated according to equation 2:

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$$N_{hb} = \sum_{h-bonds} f(\Delta R, \Delta \alpha) \times f(N_{neighb}) \times f_{pcs}$$

$f(\Delta R, \Delta \alpha)$ is a penalty function which accounts for large deviations of hydrogen bonds from ideality and is calculated according to equation 3:

$$f(\Delta R, \Delta \alpha) = f_1(\Delta R) \times f_2(\Delta \alpha)$$

5 Where: $f_1(R) = 1$ if $(R \leq TOL)$
 or $= 1 - ((R - TOL)/0.4)$ if $(R \leq 0.4 + TOL)$
 or $= 0$ if $(R > 0.4 + TOL)$

And: $f_2((\alpha)) = 1$ if $((\alpha) < 30^\circ)$
 or $= 1 - ((\Delta \alpha - 30)/50)$ if $\Delta \alpha \leq 80^\circ$
 10 or $= 0$ if $\Delta \alpha > 80^\circ$

TOL is the tolerated deviation in hydrogen bond length = 0.25Å

ΔR is the deviation of the H-O/N hydrogen bond length from the ideal value = 1.9Å

$\Delta \alpha$ is the deviation of the hydrogen bond angle $\angle_{N/O-H,O/N}$ from its idealized value of 180°

$f(N_{neighb})$ distinguishes between concave and convex parts of a protein surface and therefore
 15 assigns greater weight to polar interactions found in pockets rather than those found at the protein surface. This function is calculated according to equation 4 below:

$$f(N_{neighb}) = (N_{neighb}/N_{neighb,0})^\alpha \quad \text{where } \alpha = 0.5$$

N_{neighb} is the number of non-hydrogen protein atoms that are closer than 5Å to any given protein atom.

20 $N_{neighb,0}$ is a constant = 25

f_{pcs} is a function which allows for the polar contact surface area per hydrogen bond and therefore distinguishes between strong and weak hydrogen bonds and its value is determined according to the following criteria:

$f_{pcs} = \beta$ when $A_{polar}/N_{HB} < 10 \text{ Å}^2$
 25 or $f_{pcs} = 1$ when $A_{polar}/N_{HB} > 10 \text{ Å}^2$

A_{polar} is the size of the polar protein-ligand contact surface

N_{HB} is the number of hydrogen bonds

β is a constant whose value = 1.2

For the implementation of the modified Böhm scoring function, the contributions from ionic
 30 interactions, ΔG_{ionic} , are computed in a similar fashion to those from hydrogen bonds described above since the same geometry dependency is assumed.

The term N_{lipo} is calculated according to equation 5 below:

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$$N_{lipo} = \sum_{l,L} f(r_{lL})$$

$f(r_{lL})$ is calculated for all lipophilic ligand atoms, l , and all lipophilic protein atoms, L , according to the following criteria:

$$f(r_{lL}) = 1 \text{ when } r_{lL} \leq R1 \quad f(r_{lL}) = (r_{lL} - R1) / (R2 - R1) \text{ when } R2 < r_{lL} < R1$$

$$f(r_{lL}) = 0 \text{ when } r_{lL} \geq R2$$

$$\text{Where: } R1 = r_l^{vdw} + r_L^{vdw} + 0.5$$

$$\text{and } R2 = R1 + 3.0$$

and r_l^{vdw} is the Van der Waal's radius of atom l

and r_L^{vdw} is the Van der Waal's radius of atom L

- 10 The term N_{rot} is the number of rotatable bonds of the amino acid side chain and is taken to be the number of acyclic sp^3 - sp^3 and sp^3 - sp^2 bonds. Rotations of terminal $-CH_3$ or $-NH_3$ are not taken into account.

The final term, E_{vdw} , is calculated according to equation 6 below:

$$E_{vdw} = \epsilon_1 \epsilon_2 ((r_1^{vdw} + r_2^{vdw})^{12} / r^{12} - (r_1^{vdw} + r_2^{vdw})^6 / r^6), \text{ where:}$$

- 15 ϵ_1 and ϵ_2 are constants dependant upon atom identity

$r_1^{vdw} + r_2^{vdw}$ are the Van der Waal's atomic radii

r is the distance between a pair of atoms.

With regard to Equation 6, in one embodiment, the constants ϵ_1 and ϵ_2 are given the atom values: C: 0.245, N: 0.283, O: 0.316, S: 0.316, respectively (i.e. for atoms of Carbon,

- 20 Nitrogen, Oxygen and Sulphur, respectively). With regards to equations 5 and 6, the Van der Waal's radii are given the atom values C: 1.85, N: 1.75, O: 1.60, S: 2.00Å.

It should be understood that all predetermined values and constants given in the equations above are determined within the constraints of current understandings of protein ligand interactions with particular regard to the type of computation being undertaken herein.

- 25 Therefore, it is possible that, as this scoring function is refined further, these values and constants may change hence any suitable numerical value which gives the desired results in terms of estimating the binding energy of a protein to a ligand may be used and hence fall within the scope of the present invention.

- As described above, the scoring function is applied to data extracted from the database of side-
 30 chain conformations, atom identities, and interatomic distances. For the purposes of the present description, the number of MHC Class II molecules included in this database is 42 models plus four solved structures. It should be apparent from the above descriptions that the modular nature of the construction of the computational method of the present invention

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means that new models can simply be added and scanned with the peptide backbone library and side-chain conformational search function to create additional data sets which can be processed by the peptide scoring function as described above. This allows for the repertoire of scanned MHC Class II molecules to easily be increased, or structures and associated data to be replaced if data are available to create more accurate models of the existing alleles.

The present prediction method can be calibrated against a data set comprising a large number of peptides whose affinity for various MHC Class II molecules has previously been experimentally determined. By comparison of calculated versus experimental data, a cut off value can be determined above which it is known that all experimentally determined T-cell epitopes are correctly predicted.

It should be understood that, although the above scoring function is relatively simple compared to some sophisticated methodologies that are available, the calculations are performed extremely rapidly. It should also be understood that the objective is not to calculate the true binding energy *per se* for each peptide docked in the binding groove of a selected MHC Class II protein. The underlying objective is to obtain comparative binding energy data as an aid to predicting the location of T-cell epitopes based on the primary structure (i.e. amino acid sequence) of a selected protein. A relatively high binding energy or a binding energy above a selected threshold value would suggest the presence of a T-cell epitope in the ligand. The ligand may then be subjected to at least one round of amino-acid substitution and the binding energy recalculated. Due to the rapid nature of the calculations, these manipulations of the peptide sequence can be performed interactively within the program's user interface on cost-effectively available computer hardware. Major investment in computer hardware is thus not required.

It would be apparent to one skilled in the art that other available software could be used for the same purposes. In particular, more sophisticated software which is capable of docking ligands into protein binding-sites may be used in conjunction with energy minimization. Examples of docking software are: DOCK (Kuntz *et al.*, *J. Mol. Biol.*, 161:269-288 (1982)), LUDI (Böhm, H.J., *J. Comput Aided Mol. Des.*, 8:623-632 (1994)) and FLEXX (Rarey M., *et al.*, *ISMB*, 3:300-308 (1995)). Examples of molecular modeling and manipulation software include: AMBER (Tripos) and CHARMM (Molecular Simulations Inc.). The use of these computational methods would severely limit the throughput of the method of this invention due to the lengths of processing time required to make the necessary calculations. However, it is feasible that such methods could be used as a 'secondary screen' to obtain more accurate

calculations of binding energy for peptides which are found to be 'positive binders' via the method of the present invention.

The limitation of processing time for sophisticated molecular mechanic or molecular dynamic calculations is one which is defined both by the design of the software which makes these
5 calculations and the current technology limitations of computer hardware. It may be anticipated that, in the future, with the writing of more efficient code and the continuing increases in speed of computer processors, it may become feasible to make such calculations within a more manageable time-frame.

Further information on energy functions applied to macromolecules and consideration of the
10 various interactions that take place within a folded protein structure can be found in: Brooks, B.R., *et al.*, *J. Comput. Chem.*, 4:187-217 (1983) and further information concerning general protein-ligand interactions can be found in: Dauber-Osguthorpe *et al.*, *Proteins* 4(1):31-47(1988), which are incorporated herein by reference in their entirety. Useful background information can also be found, for example, in Fasman, G.D., ed., *Prediction of Protein*
15 *Structure and the Principles of Protein Conformation*, Plenum Press, New York, ISBN: 0-306 4313-9.

EXAMPLE 2

The interaction between MHC, peptide and T-cell receptor (TCR) provides the structural basis
20 for the antigen specificity of T-cell recognition. T-cell proliferation assays test the binding of peptides to MHC and the recognition of MHC/peptide complexes by the TCR. *In vitro* T-cell proliferation assays of the present example, involve the stimulation of peripheral blood mononuclear cells (PBMCs), containing antigen presenting cells (APCs) and T-cells. Stimulation is conducted *in vitro* using synthetic peptide antigens, and in some experiments
25 whole protein antigen. Stimulated T-cell proliferation is measured using ³H-thymidine (³H-Thy) and the presence of incorporated ³H-Thy assessed using scintillation counting of washed fixed cells.

Buffly coats from human blood stored for less than 12 hours were obtained from the National Blood Service (Addenbrooks Hospital, Cambridge, UK). Ficoll-paque was obtained from
30 Amersham Pharmacia Biotech (Amersham, UK). Serum free AIM V media for the culture of primary human lymphocytes and containing L-glutamine, 50µg/ml streptomycin, 10µg/ml gentomycin and 0.1% human serum albumin was from Gibco-BRL (Paisley, UK). Synthetic peptides were obtained from Eurosequence (Groningen, The Netherlands) and Babraham

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- Technix (Cambridge, UK). Erythrocytes and leukocytes were separated from plasma and platelets by gentle centrifugation of buffy coats. The top phase (containing plasma and platelets) was removed and discarded. Erythrocytes and leukocytes were diluted 1:1 in phosphate buffered saline (PBS) before layering onto 15ml ficoll-paque (Amersham Pharmacia, Amersham UK). Centrifugation was done according to the manufacturers recommended conditions and PBMCs were harvested from the serum+PBS/ficoll paque interface. PBMCs were mixed with PBS (1:1) and collected by centrifugation. The supernatant was removed and discarded and the PBMC pellet resuspended in 50ml PBS. Cells were again pelleted by centrifugation and the PBS supernatant discarded. Cells were resuspended using 50ml AIM V media and at this point counted and viability assessed using trypan blue dye exclusion. Cells were again collected by centrifugation and the supernatant discarded. Cells were resuspended for cryogenic storage at a density of 3×10^7 per ml. The storage medium was 90%(v/v) heat inactivated AB human serum (Sigma, Poole, UK) and 10%(v/v) DMSO (Sigma, Poole, UK). Cells were transferred to a regulated freezing container (Sigma) and placed at -70°C overnight. When required for use, cells were thawed rapidly in a water bath at 37°C before transferring to 10ml pre-warmed AIM V medium. PBMC were stimulated with protein and peptide antigens in a 96 well flat bottom plate at a density of 2×10^5 PBMC per well. PBMC were incubated for 7 days at 37°C before pulsing with ^3H -Thy (Amersham-Pharmacia, Amersham, UK). For the present study, synthetic peptides (15mers) that overlapped by 3aa increments were generated that spanned the entire sequence of IFN β . Peptide identification numbers (ID#) and sequences are given in FIGURE 5. Each peptide was screened individually against PBMC's isolated from 20 naïve donors. Two control peptides that have previously been shown to be immunogenic and a potent non-recall antigen KLH were used in each donor assay.
- The control antigens used in this study were as below:

Peptide	Sequence
C-32	Biotin-PKYVKQNTLKLAT Flu haemagglutinin 307-319
C-49	KVVDQIKKISKPVQH Chlamydia HSP 60 peptide
KLH	Whole protein from Keyhole Limpet Hemocyanin.

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Peptides were dissolved in DMSO to a final concentration of 10mM, these stock solutions were then diluted 1/500 in AIM V media (final concentration 20µM). Peptides were added to a flat bottom 96 well plate to give a final concentration of 2 and 20µM in a 100µl. The viability of thawed PBMC's was assessed by trypan blue dye exclusion, cells were then resuspended at a density of 2×10^6 cells/ml, and 100µl (2×10^5 PBMC/well) was transferred to each well containing peptides. Triplicate well cultures were assayed at each peptide concentration. Plates were incubated for 7 days in a humidified atmosphere of 5% CO₂ at 37°C. Cells were pulsed for 18-21 hours with 1µCi ³H-Thy/well before harvesting onto filter mats. CPM values were determined using a Wallac microplate beta top plate counter (Perkin Elmer). Results were expressed as stimulation indices, determined using the following formula:

Proliferation to test peptide CPM

Proliferation in untreated wells CPM

Mapping T cell epitopes in the IFNβ sequence using the T cell proliferation assay resulted in the identification of two immunogenic regions R1 and R2. This was determined by T cell proliferation in six donors that responded to peptides in one or more of these regions. Regions 1 and 2 induce T-cell proliferation in certain individuals. The cumulative response data for the responding individuals is depicted in FIGURE 6, and data from individual responders given in FIGURE 7. The epitope data for IFNβ and indicating R1 and R2 and the individual peptide/donor responses is depicted in FIGURES 8 and 9.

EXAMPLE 3

A number of modified IFNβ molecules were made using conventional recombinant DNA techniques. A wild-type IFNβ gene was used both as a control reagent, and a template from which to derive modified genes by site-directed mutagenesis. Wild-type and modified genes were inserted into a eukaryotic expression vector and the recombinant IFNβ proteins expressed as fusion protein with the human immunoglobulin constant region domain. Recombinant proteins were prepared from transiently transfected human embryonic kidney cells and assayed as detailed in EXAMPLE 4.

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In order to obtain expression from human embryonic kidney cells, the wild-type human IFN β gene was obtained from the ATCC (ATCC accession # 31902) and PCR cloned into vector pd-Cs [Lo, et al (1998), *Protein Engineering* 11: 495]. The pd-Cs vector directs the expression of a fusion protein containing the human immunoglobulin constant region domain.

5 The pd-Cs vector containing the wild-type IFN β gene was termed pdCs IFN β WT.

Single or multiple codon mutations to generate modified IFN β genes was conducted by mutagenic PCR using pdCsIFN β WT as a template. Overlap PCR was used to combine the two mutated halves of the interferon sequence. The PCR product of 503 bp was digested with
10 XmaI and BamHI, purified using a Qiagen gel extraction kit and transferred into prepared pd-Cs from which the IFN β sequence had been removed using XmaI and BamHI. A positive clone was selected and the IFN β sequence confirmed by sequence analysis.

Mutagenesis was conducted using flanking primers OL575 and OL576 in separate reactions in
15 combination with specific mutagenic (mis-matched) primers and the pdCs IFN β WT template DNA.

OL575 (XmaI)

5' CTCCCTGTCCCCGGGTATGAG 3'

OL576 (XhoI/BamHI)

20 5' -CTTATCATGTCTGGATCCCTCGAG-3'

Reactions were conducted using Expand HI Fidelity PCR reagents (Roche, GmbH) and reaction conditions specified by the following cycle:

25 94°C/2' + 25 Cycles @ 94°C/30", 60°C/30", 72°C/30" + 72°C/10'

The products of the separate reactions were joined by PCR in a reaction driven by primers OL575 and OL576 using 15 cycles of PCR as above.

30 PCR products were gel purified using commercially available kit systems (Qiagen gel extraction kit). The desired clones were digested with BamHI and XmaI and the purified product ligated into a prepared pd-Cs vector. Cloning was conducted using *E.coli* XL1-Blue cells (Stratagene Europe) and culture conditions recommended by the supplier. Sequence

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confirmation was conducted on all final vector preparations using OL575 and OL576 as sequencing primers.

Expression of modified IFN β -1a human IgFc fusion proteins was achieved using HEK293 human embryonic kidney cell line as the expression host. All DNA for transfection was prepared using the high purity QIAGEN midi-prep system and instructions provided by the supplier (QIAGEN, Crawley, UK). DNA is filter sterilised prior to use and quantified by measurement of the A₂₆₀. Concentrations were adjusted to 0.5-1.0 ug/ul.

For transient expression, HEK293 were grown using DMEM L-Glutamax medium (Invitrogen, Paisley, UK) supplemented with 10% FBS and 250 ug/ml Geneticin. Prior to transfection, cells were collected by treatment with trypsin and washed using PBS. After 2 cycles of washing cells are taken into fresh medium at a density of 4×10^5 cells/ml, and plated into multi-well dishes pre-treated with poly-l-lysine to ensure good cell adhesion. Typically, 2×10^5 cells are added to each well of a 48 well plate and the plates incubated overnight at 37°C/5%CO₂.

Prior to transfection, the medium is replaced in each well and the transfection mixes added. Transfection is conducted using the lipofectamine reagent and instructions provided by the supplier (Invitrogen, Paisley, UK). Briefly, transfection mixes are prepared containing lipofectamine, OPTI-MEM (Invitrogen, Paisley, UK) and 0.8 ug DNA per well for each expression vector construct. Transfection mixes are added to the cells and the cells incubated for 4-6 hours. The medium is replaced with 0.5 ml fresh media and the cells incubated at 37°C/5%CO₂. Samples were taken after 48 hours for analysis by both anti-Fc ELISA and Daudi cell proliferation assay. The media was harvested after 7 days and stored at 4°C for further analysis as above.

The medium is assayed for the presence of IFN β using an ELISA detecting the human immunoglobulin constant region domain of the IFN β -fusion protein. For this assay a mouse anti-human IgG Fc preparation (Sigma, Poole, UK) is used as a capture reagent. The IFN β -HuFc fusion is quantitated with reference to a standard curve generated using a dilution series of a reference human IgG preparation (Sigma). Bound IFN β -Fc fusion or the reference protein is detected using an anti-human IgG peroxidase conjugate (Sigma) and Sigma OPD colourimetric substrate.

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Following estimation of the amount of IFN β in the HEK.293 conditioned medium, the conditioned medium is used directly to test the functional activity of the modified IFN β using the anti-proliferation assay as detailed in EXAMPLE 4.

5 EXAMPLE 4

Modified interferon molecules of the present invention were tested for their ability to inhibit the growth of human B cell lymphoma line Daudi. The method is broadly as described previously [Mark, D.F. et al (1984) *Proc. Natl. Acad. Sci. USA* 81: 5662-5666] and involves incubation of Daudi cells with the test interferon. The anti-proliferative effect of the test

10 molecule is measured using a soluble dye substance that undergoes a colour change in the presence of proliferating cells. The induced colour change is measured in a spectrophotometer and any anti-proliferation effect is computed with reference to the colour change recorded in non-treated control cells and cells treated with a standard interferon preparation.

Briefly, Daudi cells (ATCC # CCL-213) were cultured RPMI 1640 Media supplemented with

15 100 units/ml Penicillin/ 100 ug/ml Streptomycin and 2 mM L-Glutamine and 20% Fetal Bovine Serum (FBS). All media and supplements were from Gibco (Paisley, UK). The day before assay, cells are replaced into fresh medium at a density 0.9×10^6 /ml and next day replaced into fresh medium as above except containing 10%(v/v) FBS. The cell density is adjusted to be 2×10^5 cells/ml.

20 The test and control interferon preparations are diluted into RPMI containing 10% FBS. Dilutions are made into 96-well flat bottom plates to contain 100ul/ well and all samples are set up in triplicate. Typically doubling dilution series are set out across each plate. Positive control wells are also included in triplicate with a starting concentration of the interferon standard (R&D Systems, Abingdon, UK) at 20000 pg/ml. Control wells containing 100ul

25 media alone (no interferon) are also included. 100ul of the cells are added to each well, and the plates incubated for 72 hours at 37°C, 5% CO₂.

Proliferation is assessed using Aqueous One reagent system and the suppliers recommended protocol (Promega, Southampton, UK). Briefly, 40 ul of the Aqueous One reagent is added to all wells and the substrate mixed. Plates are incubated at 37°C for one hour, and then

30 transferred to the plate reading instrument for determination of the light absorbance. Readings are taken at 490nm. Average absorbance at 490 nm is plotted on the Y axis versus concentrations of interferon standard added along the X axis. Interferon concentration is determined using an ELISA technique as detailed in EXAMPLE 3. For each mutant, the

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IFN β -1a concentration required to achieve 50% inhibition of cell growth (EC_{50}) was determined from the plot of absorbance versus concentration.

Results of such an analysis according to the above method for a number of modified IFN β -1a molecules are depicted in FIGURE 10. The results indicate retained anti-proliferative
5 properties in the presence of amino acid substitutions within the IFN β sequence.

Patent Claims

1. A modified molecule having the biological activity of human interferon beta (INF β) and being substantially non-immunogenic or less immunogenic than any non-modified molecule having the same biological activity when used *in vivo*.
5
2. A molecule according to claim 1, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule and / or by reduction in numbers of MHC allotypes able to bind peptides
10 derived from said molecule.
3. A molecule according to claim 2, wherein one T-cell epitope is removed.
4. A molecule according to any of the claims 2 – 4, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to
15 stimulate or bind T-cells via presentation on MHC class II.
5. A molecule according to claim 4, wherein said ligands or peptide sequences are 13mer or 15mer peptides.
20
6. A molecule according to claim 5, wherein said peptide sequences are selected from the group as depicted in Figure 1.
7. A molecule according to any of the claims 2 – 6, wherein 1 – 9 amino acid residues in
25 any of the originally present T-cell epitopes are altered.
8. A molecule according to claim 7, wherein one amino acid residue is altered.
9. A molecule according to claim 7 or 8, wherein the alteration of the amino acid residues
30 is substitution of originally present amino acid(s) residue(s) by other amino acid residue(s) at specific position(s).

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10. A molecule according to claim 9, wherein one or more of the amino acid residue substitutions are carried out as indicated in Figure 2.
11. A molecule according to claim 10, wherein additionally one or more of the amino acid residue substitutions are carried out as indicated in Figure 3 for the reduction in the number of MHC allotypes able to bind peptides derived from said molecule.
12. A molecule according to claim 9, wherein one or more amino acid substitutions are carried as indicated in Figure 3.
13. A molecule according to claim 7 or 8, wherein the alteration of the amino acid residues is deletion of originally present amino acid(s) residue(s) at specific position(s).
14. A molecule according to claim 7 or 8, wherein the alteration of the amino acid residues is addition of amino acid(s) at specific position(s) to those originally present.
15. A molecule according to any of the claims 7 to 14, wherein additionally further alteration is conducted to restore biological activity of said molecule.
16. A molecule according to claim 15, wherein the additional further alteration is substitution, addition or deletion of specific amino acid(s).
17. A modified molecule according to any of the claims 7 – 16, wherein the amino acid alteration is made with reference to an homologous protein sequence.
18. A modified molecule according to any of the claims 7 – 16, wherein the amino acid alteration is made with reference to *in silico* modeling techniques.
19. A modified molecule according to any of the claims 7-16, wherein the amino acid alteration is made with reference to stimulation or binding of T cells by INF β derived peptides or INF β protein.

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20. A modified molecule having the biological activity of human interferon beta (INF β) and being substantially non-immunogenic or less immunogenic than any non-modified molecule and having the biological activity of a human INF β when used *in vivo*, obtainable by alteration of one or more amino acids in the primary sequence by (i)
5 removing one or more T-cell epitopes derived from the originally non-modified molecule and being MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on MHC class II, and / or (ii) by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule, wherein said modified molecule comprises alterations which are made at one or more
10 positions within following strings of contiguous amino acid residues of said primary sequence derived from the INF β wild-type:
(a) QFQKEDAALTIYEMLQNIFAIFRQ (R1),
(b) RYYGRILHYLKAKEYSHCAWT (R2),
- 15 21. A molecule according to claim 20, wherein said alteration is substitution of 1 – 9 amino acid residues.
22. A molecule according to claim 21, wherein said substitution is conducted at one or more amino acid residues from the strings R1 and R2.
- 20 23. A molecule according to claim 21, wherein said substitution is conducted at one or more amino acid residues from the string R1
24. A molecule according to claim 21, wherein said substitution is conducted at one or more
25 amino acid residues from the string R2.
25. A molecule according to any of the claims 20 – 24, wherein additionally one or more substitutions of amino acid residues outside the sequence strings R1 or R2 are conducted.
- 30 26. A molecule according to any of the claims 20 – 25, comprising an amino acid residue substitution made at one or more positions in the wild-type molecule:
50, 57, 59, 60, 62, 63, 66, 67, 69, 70, 125, 126, 129, 130, 132, 133, 138.

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27. A molecule according to claim 26, wherein said substitution is made at one or more positions selected from 50, 57, 59, 60, 62, 63, 66, 67, 69 or 70.
28. A molecule according to claim 26, wherein said substitution is made at one or more positions selected from 125, 126, 129, 130, 132, 133 or 138.
29. A molecule of claim 26, wherein said substitution is made at positions 57 and 67.
30. A molecule according to claim 29 wherein said substitutions are L57A and F67H.
31. A molecule according to claim 30 wherein additional substitutions are made at one or more positions selected from the group 50, 59, 60, 62, 63, 66, 69, 70, 125, 126, 129, 130, 132, 133, 138.
32. A molecule according to claim 27, wherein said substitution is selected from F50A, L57A, I59A, Y60N, M62A, L63A, I66T, F67H, I69A, F70A.
33. A molecule according to claim 28, wherein said substitution is selected from Y125A, Y126A, I129A, L130A, Y132S, L133A, Y138H, Y138A.
34. A molecule according to claim 31 wherein said additional substitutions are selected from the group F50A, I59A, Y60N, M62A, L63A, I66T, I69A, F70A, Y125A, Y126A, I129A, L130A, Y132S, L133A, Y138H, Y138A.
35. A modified molecule having the biological activity of human interferon beta (INF β) and being substantially non-immunogenic or less immunogenic than any non-modified molecule having the same biological activity when used *in vivo*, obtainable by substitution of one or more amino acids in the primary sequence by (i) removing one or more T-cell epitopes derived from the originally non-modified molecule and being MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on MHC class II, and / or (ii) by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule, wherein said substitution is made at one or more positions in a wild-type molecule INF β corresponding to at least

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one of the groups selected from:

- (i) L57A, F67H,
- (ii) F50A, L57A, I59A, Y60N, M62A, L63A, I66T, F67H, I69A, F70A and
- (iii) Y125A, Y126A, I129A, L130A, Y132S, L133A, Y138H, Y138A
- 5 (iv) any position within sequence R1, and
- (v) any position within sequence R2.

36. A molecule of claim 35, whereby one or more of the following substitutions are made within sequence R1: F50A, L57A, I59A, Y60N, M62A, L63A, I66T,
10 F67H, I69A, F70A.

37. A molecule of claim 35, whereby one or more of the following substitutions are made within sequence R2: Y125A, Y126A, I129A, L130A, Y132S, L133A,
Y138H, Y138A

15

38. A modified human interferon beta (INF β) having reduced immunogenicity consisting of the following sequence:

MSYNLLGFLQRSSNFQX⁰QKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQOX¹QKEDAAX²TX³X⁴E
X⁵X⁶QNX⁷X⁸AX⁹X¹⁰RQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLH
20 LKRYYGRIHLHYLKAKEYSHCAWTIVRVEILRNIFYFINRLTGYLRLN

wherein X⁰ is C, S;

X¹ is F, A;

X² is L, A;

X³ is I, A;

25 X⁴ is Y, N;

X⁵ is M, A;

X⁶ is L, A;

X⁷ is I, T;

X⁸ is F, H;

30 X⁹ is I, A and, ;

X¹⁰ is F, A;

whereby simultaneously X¹ = F, X² = L, X³ = I, X⁴ = Y, X⁵ = M, X⁶ = L, X⁷ = I, X⁸ = F,
X⁹ = I and X¹⁰ = F are excluded.

39. A modified human interferon beta (INF β) having reduced immunogenicity consisting of the following sequence:

MSYNLLGFLQRSSNFQX⁰QKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAALTIYEML
 QNIFAIFRQDSSSTGWNENITIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHLKRX¹X²GRX³X⁴HX⁵X⁶KAKEX⁷SHCAWTIVRVEILRNIFYFINRLTGYLRN,

wherein X⁰ is C, S;

X¹ is Y, A;

X² is Y, A;

X³ is I, A;

X⁴ is L, A;

X⁵ is Y, S;

X⁶ is L, A and

X⁷ is Y, H, A;

whereby simultaneously X¹ = Y, X² = Y, X³ = I, X⁴ = L, X⁵ = Y, X⁶ = L and X⁷ = Y are excluded.

40. A modified INF β sequence according to claim 38 containing in addition substitutions according to claim 39.

41. A modified INF β sequence according to any of claims 38- 40, wherein additional substitutions are made.

42. A modified INF β sequence according to claim 41, wherein additional substitutions are made at one or more positions within partial sequence R1 and / or R2, wherein R1 and R2 are defined as specified in claim 20.

43. A DNA sequence coding for a modified INF β of any of the claims 1 – 42.

44. A pharmaceutical composition comprising a modified molecule having the biological activity of INF β as defined in any of the above-cited claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient.

45. A method for manufacturing a modified molecule having the biological activity of $\text{INF}\beta$ as defined in any of the claims of the above-cited claims comprising the following steps:
- (i) determining the amino acid sequence of the polypeptide or part thereof.
 - (ii) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays;
 - (iii) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays, or by binding of peptide-MHC complexes to T-cells.
 - (iv) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties; and
 - (v) optionally repeating steps (ii) – (iv).
46. A method of claim 45, wherein step (iii) is carried out by substitution, addition or deletion of 1 – 9 amino acid residues in any of the originally present T-cell epitopes.
47. A method of claim 45, wherein the alteration is made with reference to an homologous protein sequence and / or *in silico* modeling techniques.
48. A method of any of the claims 45 – 47, wherein step (ii) is carried out by the following steps: (a) selecting a region of the peptide having a known amino acid residue sequence; (b) sequentially sampling overlapping amino acid residue segments of predetermined uniform size and constituted by at least three amino acid residues from the selected region; (c) calculating MHC Class II molecule binding score for each said sampled segment by summing assigned values for each hydrophobic amino acid residue side chain present in said sampled amino acid residue segment; and (d) identifying at least one of said segments suitable for modification, based on the calculated MHC Class II molecule binding score for that segment, to change overall MHC Class II binding score for the peptide without substantially the reducing therapeutic utility of the peptide.

49. A method of claim 48, wherein step (c) is carried out by using a Böhm scoring function modified to include 12-6 van der Waal's ligand-protein energy repulsive term and ligand conformational energy term by (1) providing a first data base of MHC Class II molecule models; (2) providing a second data base of allowed peptide backbones for said MHC Class II molecule models; (3) selecting a model from said first data base; (4) selecting an allowed peptide backbone from said second data base; (5) identifying amino acid residue side chains present in each sampled segment; (6) determining the binding affinity value for all side chains present in each sampled segment; and repeating steps (1) through (5) for each said model and each said backbone.
50. A peptide molecule consisting of 13 consecutive amino acid residues having a potential MHC class II binding activity and created from the primary sequence of non-modified INF β , selected from the group as depicted in Figure 1.
51. A peptide molecule consisting of 12 or more consecutive amino acid residues having a potential MHC class II binding activity and created from the primary sequence of non-modified INF β , selected from any of the groups of partial sequences R1 or R2 as specified in claim 20.
52. A peptide molecule according to claim 51 selected from Figure 8.
53. A peptide sequence consisting of at least 9 consecutive amino acid residues selected from the T-cell epitope peptides as specified in claims 50 – 52.
54. A peptide molecule consisting of 9 – 15 consecutive amino acid residues, having a potential MHC class II binding activity and created from the primary sequence of non-modified INF β , whereby said molecule has a stimulation index of at least 1.8 and preferably 2 or greater, in a biological assay of cellular proliferation wherein said index is taken as the value of cellular proliferation scored following stimulation by a peptide and divided by the value of cellular proliferation scored in control cells not in receipt peptide and wherein cellular proliferation is measured by any suitable means.

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55. Use of a peptide according to any of the claims 50 – 54, for the manufacture of $\text{INF}\beta$ having substantially no or less immunogenicity than any non-modified molecule with the same or acceptably reduced degree of biological activity when used *in vivo*.
- 5 56. Use of a peptide or peptide sequence derived from any of the sequences R1 or R2 of claim 20 for the purpose of vaccination of patients to reduce immunogenicity to $\text{INF}\beta$ *in vivo*.
57. A pharmaceutical composition comprising a synthetic peptide sequence derived from any of R1 or R2 of claim 20, optionally together with a pharmaceutically acceptable
10 carrier, diluent or excipient.

FIGURE 1

Peptide sequences in human INF β with potential human MHC class II binding activity.

MSYNLLGFLQRSS,	YNLLGFLQRSSNF,	NLLGFLQRSSNFQ,
LLGFLQRSSNFQC,	LGFLQRSSNFQCQ,	GFLQRSSNFQCQK,
RSSNFQCQKLLWQ,	SNFQCQKLLWQLN,	QKLLWQLNGRLEY,
KLLWQLNGRLEYC,	LLWQLNGRLEYCL,	WQLNGRLEYCLKD,
GRLEYCLKDRMNF,	LEYCLKDRMNFDI,	YCLKDRMNFDIPE,
DRMNFDIPEEIKQ,	MNFDIPEEIKQLQ,	FDIPEEIKQLQQF,
DIPEEIKQLQQFQ,	EEIKQLQQFQKED,	KQLQQFQKEDAAL,
QLQQFQKEDAALT,	QQFQKEDAALTIY,	FQKEDAALTIYEM,
KEDAALTIYEMLQ,	AALTIYEMLQNI,	LTIIYEMLQNIFAI,
TIYEMLQNIFAIF,	IYEMLQNIFAIFR,	YEMLQNIFAIFRQ,
EMLQNIFAIFRQD,	QNIFAIFRQDSSS,	NIFAIFRQDSSST,
FAIFRQDSSSTGW,	AIFRQDSSSTGWN,	TGWNETIVENLLA,
GWNETIVENLLAN,	WNETIVENLLANV,	ETIVENLLANVYH,
TIVENLLANVYHQ,	ENLLANVYHQINH,	NLLANVYHQINHL,
ANVYHQINHLKTV,	NVYHQINHLKTVL,	HQINHLKTVLEEK,
NHLKTVLEEKLEK,	KTVLEEKLEKEDF,	TVLEEKLEKEDFT,
VLEEKLEKEDFTR,	EKLEKEDFTRGKL,	KLEKEDFTRGKLM,
LEKEDFTRGKLMS,	EKEDFTRGKLMSS,	EDFTRGKLMSSLH,
TRGKLMSSSLHLKR,	GKLMSSSLHLKRY,	KLMSSSLHLKRYG,
LMSSLHLKRYYGR,	SSLHLKRYYGRIL,	LHLKRYYGRILHY,
KRYYGRILHYLKA,	RYYGRILHYLKAK,	YYGRILHYLKAKE,
GRILHYLKAKEYS,	RILHYLKAKEYSH,	LHYLKAKEYSHCA,
HYLKAKEYSHCAW,	YLKAKEYSHCAWT,	KEYSHCAWTIVRV,
HCAWTIVRVEILR,	CAWTIVRVEILRN,	WTIVRVEILRNFY,
TIVRVEILRNFYF,	IVRVEILRNFYFI,	VRVEILRNFYFIN,
VEILRNFYFINRL,	EILRNFYFINRLT,	LRNFYFINRLTGY,
RNFYFINRLTGYL,	NFYFINRLTGYLR,	FYFINRLTGYLRN

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FIGURE 2

Residue #	WT Residue	Substitutions												
3	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
6	L	N	P	Q	R	S	T							
8	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
9	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
15	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
20	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
21	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
22	W	A	C	D	E	G	H	K	N	P	Q	R	S	T
24	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
28	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
30	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
32	L	A	C	D	E	G	H	K	N	P	Q	R	S	
36	M	A	C	D	E	G	H	K	N	P	Q	R	S	T
38	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
40	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
44	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
47	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
50	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
57	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
60	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
62	M	A	C	D	E	G	H	K	N	P	Q	R	S	T
63	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
66	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
67	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
69	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
70	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
79	W	A	C	D	E	G	H	K	N	P	Q	R	S	T
83	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
84	V	A	C	D	E	K	N	P	Q	R	S	T		
87	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
88	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
91	V	A	C	D	E	G	H	K	N	P	Q	R	S	T
92	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
95	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
98	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
101	V	A	C	D	E	G	H	K	N	P	Q	R	S	T
102	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
106	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
111	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
116	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
117	M	A	C	D	E	G	H	K	N	P	Q	R	S	T
120	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
122	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
125	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
126	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
129	I	A	C	D	E	G	H	K	N	P	Q	R	S	T

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FIGURE 2 cont...

Residue #	WT Residue	Substitutions												
130	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
132	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
133	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
138	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
143	W	A	C	D	E	G	H	K	N	P	Q	R	S	T
145	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
146	V	A	C	D	E	G	H	K	N	P	Q	R	S	T
148	V	A	C	D	E	G	H	K	N	P	Q	R	S	T
150	I	A	C	D	E	G	H	K	N	P	Q	R	S	T
151	L	A	C	D	E	G	H	K	N	P	Q	R	S	T
154	F	A	C	D	E	G	H	K	N	P	Q	R	S	T
155	Y	A	C	D	E	G	H	K	N	P	Q	R	S	T
156	F	A	C	D	E	G	H	K	N	P	Q	R	S	T

FIGURE 3

Residue #	WT residue	Substitution											
6	L	A	C	D	E	F	G	H	I	K	V	W	Y
9	L	F	I	M	V	W	Y						
10	Q	A	C	G	I	P							
11	R	A	C	G	P								
12	S	P	T										
13	S	A	C	G	P								
14	N	D	H	P									
15	F	M	W	Y									
16	Q	A	C	G	P								
17	C	D	E	H	K	N	P	Q	R	S	T		
18	Q	H	P	T									
19	K	A	C	G									
20	L	W	Y										
21	L	M	W	Y									
23	Q	H	P	T									
24	L	F	I	M	V	W	Y						
25	N	A	C	G	P								
26	G	H	T										
28	L	F	I	M	V	W	Y						
29	E	A	C	G	H	P	W						
30	Y	M											
31	C	D	E	H	K	N	P	Q	R	S	T		
32	L	F	I	M	V	W	Y						
33	K	A	C	G	H	P	T						
34	D	A	C	G	P	T							
35	R	A	C	H	P	T							
36	M	F	I	M	V	W	Y						
37	N	A	C	G	H	P	W						
38	F	I	M	V	W	Y							
39	D	A	C	G	P								
42	E	A	C	G	P								
43	E	H	P										
44	I	M	W										
45	K	A	C	G	P								
46	Q	P	T										
47	L	M	W	Y									
48	Q	A	C	G	P								
50	F	M	W										
51	Q	A	C	G	P								
52	K	A	C	G	H	P	T						
53	E	H	P	T									
54	D	A	C	G	P								
55	A	C	D	E	G	H	K	N	P	Q	R	S	T
56	A	D	E	G	H	K	N	P	Q	R	S	T	
57	L	M	V	W	Y								
58	T	A	C	G	P								
61	E	A	C	G	P								

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FIGURE 3 cont...

Residue #	WT residue	Substitution											
62	M W	Y											
63	L F	M	V	W	Y								
64	Q A	C	G	P									
65	N H	P	T										
66	I M	W	Y										
67	F M	W	Y										
68	A D	E	F	H	K	N	P	Q	R	S	T		
69	I M												
70	F M	W											
71	R A	C	G	P									
72	Q A	C	G	P	T								
73	D A	C	G	P	T								
74	S A	C	G	P	T								
75	S P	T											
76	S A	C	G	P									
77	T A	C	G	P									
78	G D	E	H	K	N	P	Q	R	S	T			
80	N A	C	G	P									
81	E A	C	G	P									
82	T P												
84	V L	M	W	Y									
85	E P	T											
86	N A	C	G	P									
87	L F	I	M	V	W	Y							
89	A H	P											
90	N T												
100	T H												
102	L I	M	V	W	Y								
103	E A	C	G	P	T								
104	E P												
105	K H	Q	S	T									
106	L M	W	Y										
107	E H												
108	K H	N	Q	S	T								
109	E P	T											
110	D H	Q	S	T									
111	F M	W	Y										
112	T A	C	G	P									
114	G H	K	N	P	Q	S	T						
116	L F	I	M	V	W	Y							
118	S A	C	G	P									
119	S P	T											
120	L M	W	Y										
121	H												
122	L W	Y											
123	K A	C	G	P	T								
124	R A	C	G	P	T								

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FIGURE 3 cont...

Residue #	WT residue	Substitution
127	G P	
128	R H P T	
129	I W Y	
130	L W Y	
133	L I M V W Y	
134	K A C G H P	
135	A C G H K N P Q R S T	
136	K P T	
137	E A C G P T	
139	S P T	
140	H A C G P	
141	C D E H K N P Q R S T	
145	I W	
148	V I L W Y	
151	L F M V W Y	
152	R A C G P W Y	
153	N A C G P T	
154	F M	
156	F I M W Y	
157	I T	
158	N A C F G I L M P V W Y	
159	R D F H I K N P Q S T V W Y	
160	L D E F G H I K N P Q R S T Y	
161	T D E F H I L N P Q S V W Y	
162	G D E F H I K N P Q R S T V W Y	
164	L A C D E F G H I K M N P Q R S T V W Y	

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FIGURE 4

Substitution			Epitope Region
WT	#	MUT	
Leu	57	Ala	R1
Tyr	60	Asn	R1
Met	62	Ala	R1
Leu	63	Ala	R1
Ile	66	Thr	R1
Phe	67	His	R1
Ile	69	Ala	R1
Ile	59	Ala	R1
Phe	50	Ala	R1
Phe	70	Ala	R1
Tyr	125	Ala	R2
Tyr	126	Ala	R2
Ile	129	Ala	R2
Leu	130	Ala	R2
Tyr	132	Ser	R2
Leu	133	Ala	R2
Tyr	138	His	R2
Tyr	138	Ala	R2

FIGURE 5

Peptide ID Number	IFN β -1a; 15mer sequence	Position of 1st peptide residue within IFN β -1a sequence	Peptide ID Number	IFN β 1a; 15mer sequence	Position of 1st peptide residue within IFN β 1a sequence
1	MSYNLLGFLQRSSNF	1	28	TIVENLLANVYHQIN	82
2	NLLGFLQRSSNFQCQ	4	29	ENLLANVYHQINHLK	85
3	GFLQRSSNFQCQKLL	7	30	LANVYHQINHLKTVL	88
4	QRSSNFQCQKLLWQL	10	31	VYHQINHLKTVLEEK	91
5	SNFQCQKLLWQLNGR	13	32	QINHLKTVLEEKLEK	94
6	QCQKLLWQLNGRLEY	16	33	HLKTVLEEKLEKEDF	97
7	KLLWQLNGRLEYCLK	19	34	TVLEEKLEKEDFTRG	100
8	WQLNGRLEYCLKDRM	22	35	EEKLEKEDFTRGKLM	103
9	NGRLEYCLKDRMNFD	25	36	LEKEDFTRGKLMSSL	106
10	LEYCLKDRMNFDIPE	28	37	EDFTRGKLMSSLHLK	109
11	CLKDRMNFDIPEEIK	31	38	TRGKLMSSLHLKRY	112
12	DRMNFDIPEEIKQLQ	34	39	KLMSSLHLKRYYGRI	115
13	NFDIPEEIKQLQQFQ	37	40	SSLHLKRYYGRI LHY	118
14	IPEEIKQLQQFQKED	40	41	HLKRYYGRI LHYLKA	121
15	EIKQLQQFQKEDAAL	43	42	RYYGRI LHYLKAKEY	124
16	QLQQFQKEDAALTIY	46	43	GRILHYLKAKEYSHC	127
17	QFQKEDAALTIYEML	49	44	LHYLKAKEYSHCAWT	130
18	KEDAALTIYEMLQNI	52	45	LKAKEYSHCAWTIVR	133
19	AALTIYEMLQNIFAI	55	46	KEYSHCAWTIVRVEI	136
20	TIYEMLQNIFAI FRQ	58	47	SHCAWTIVRVEILRN	139
21	EMLQNIFAI FRQDSS	61	48	AWTIVRVEILRN FYF	142
22	QNIFAI FRQDSSSTG	64	49	IVRVEILRN FYFINR	145
23	FAI FRQDSSSTGWNE	67	50	VEILRN FYFINRLTG	148
24	FRQDSSSTGWN ETIV	70	51	LRN FYFINRLTGYLR	151
25	DSSSTGWN ETIVENL	73			
26	STGWN ETIVENLLAN	76			
27	WNETIVENLLANVYH	79			

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FIGURE 6a

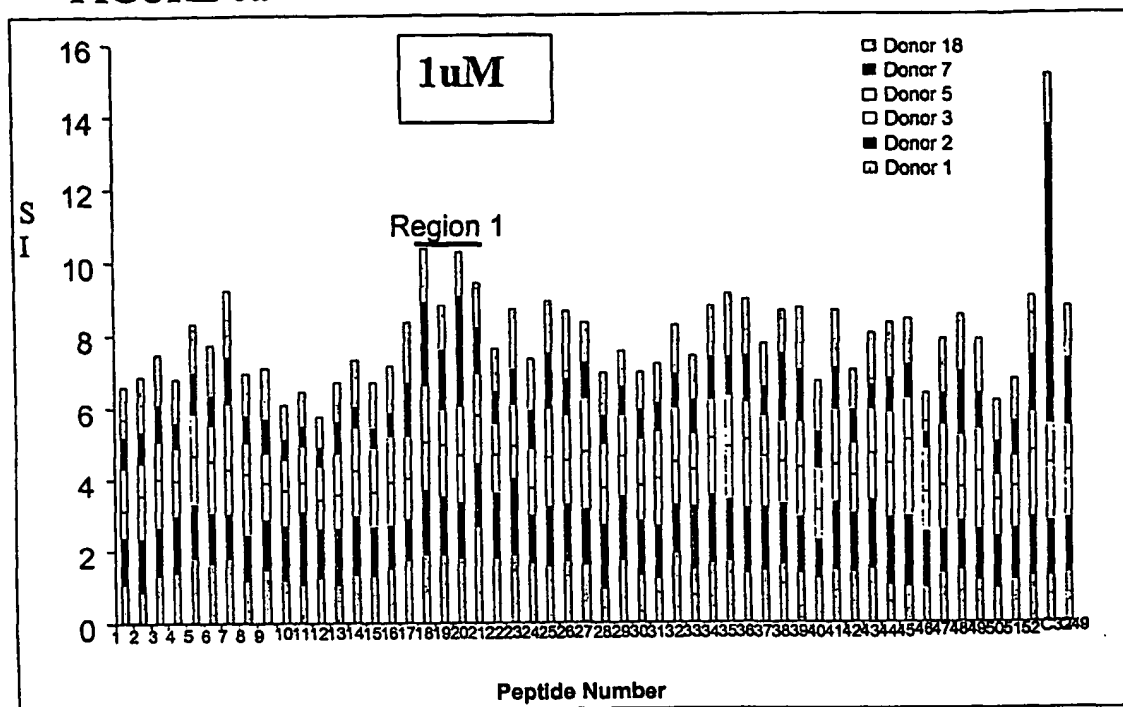
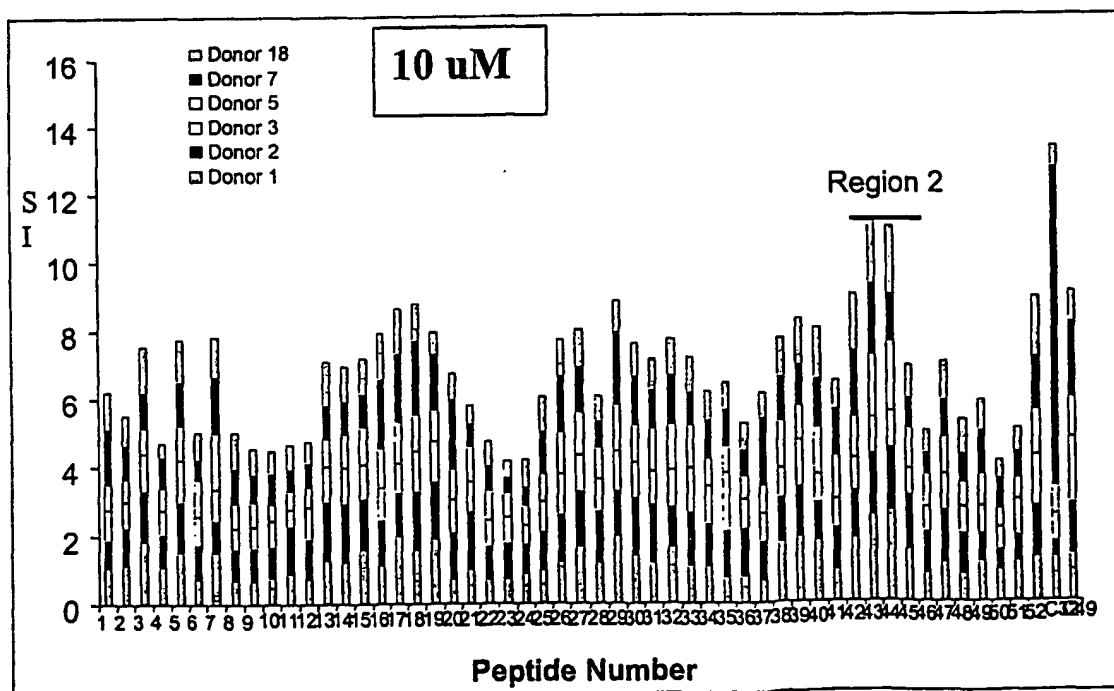
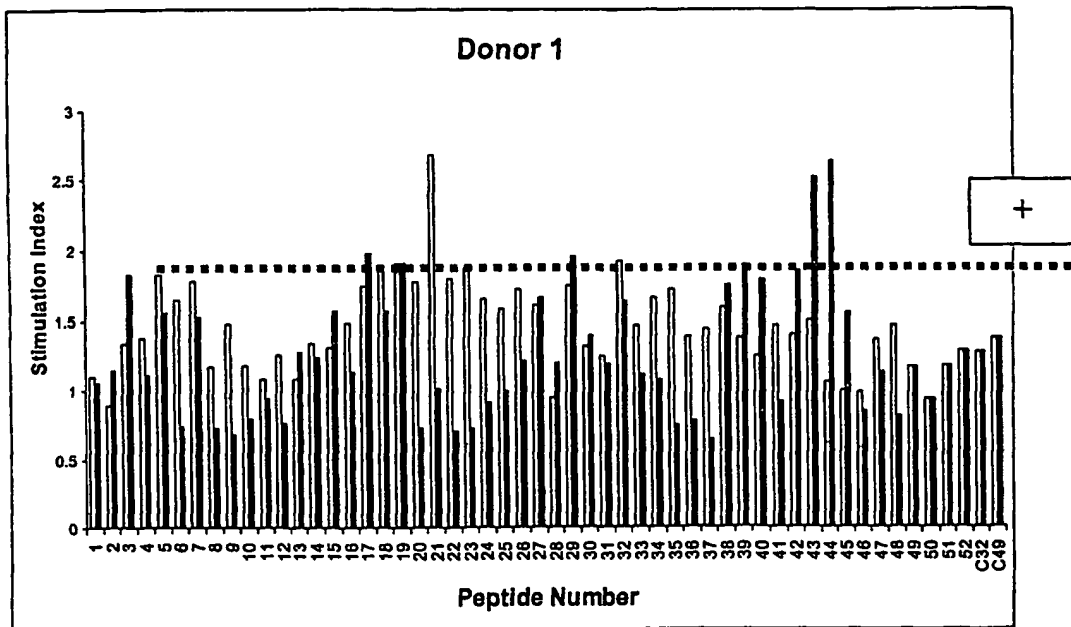
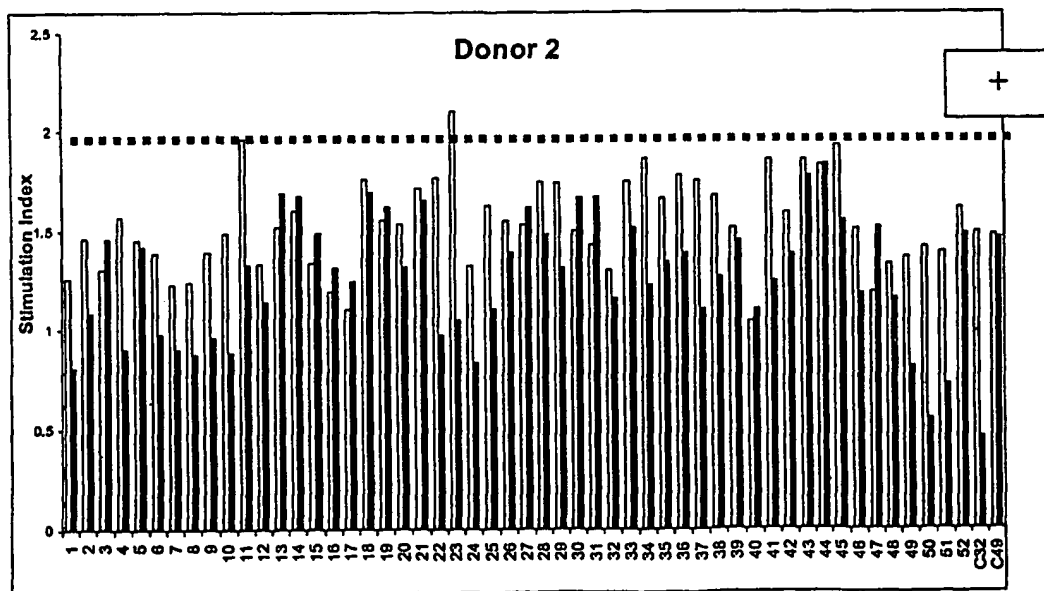


FIGURE 6b



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FIGURE 7a**FIGURE 7b**

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FIGURE 7c

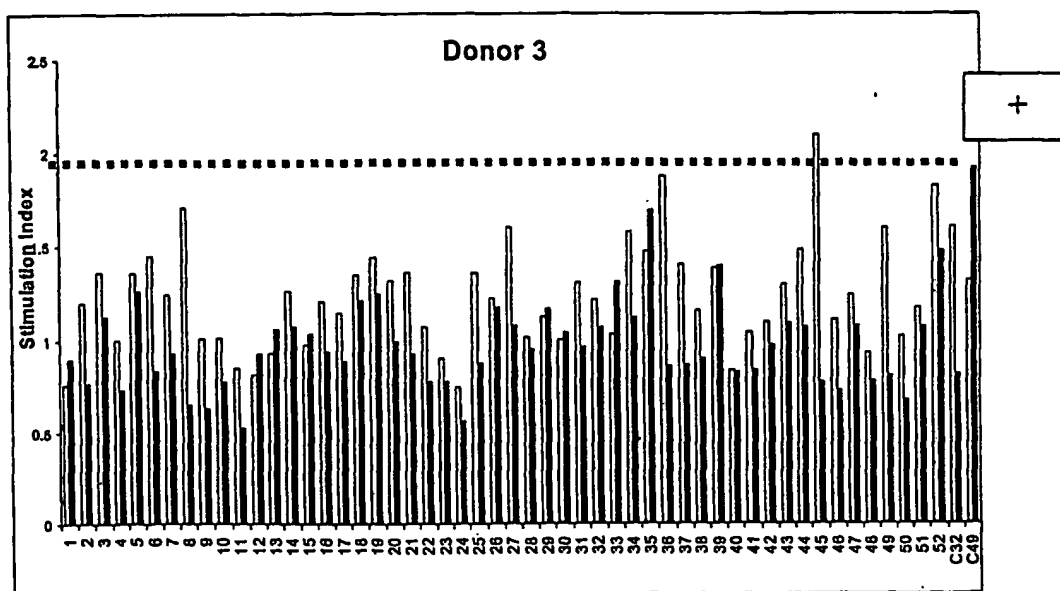
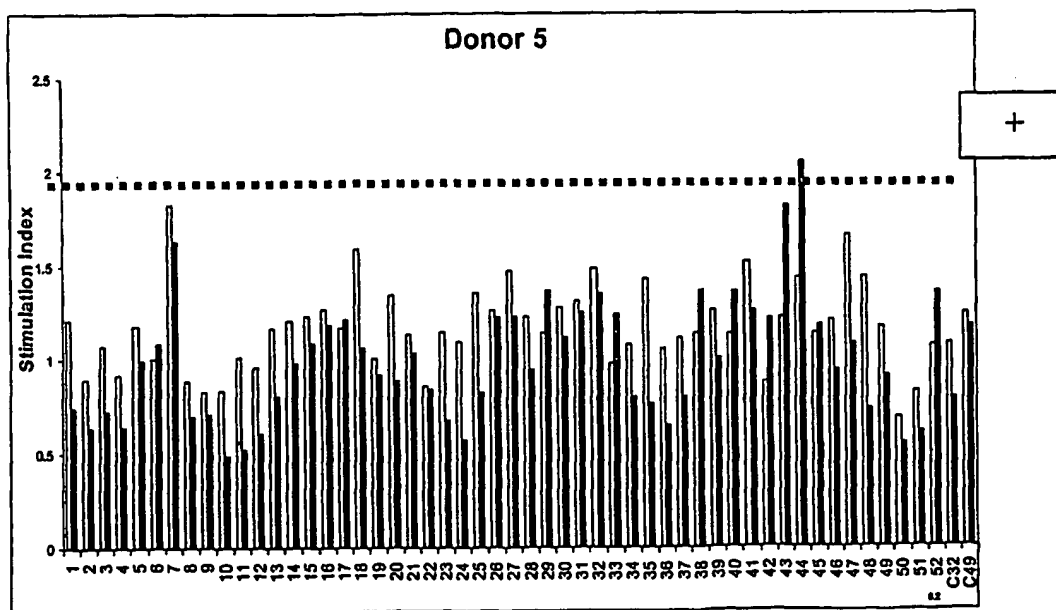


FIGURE 7d



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FIGURE 7e

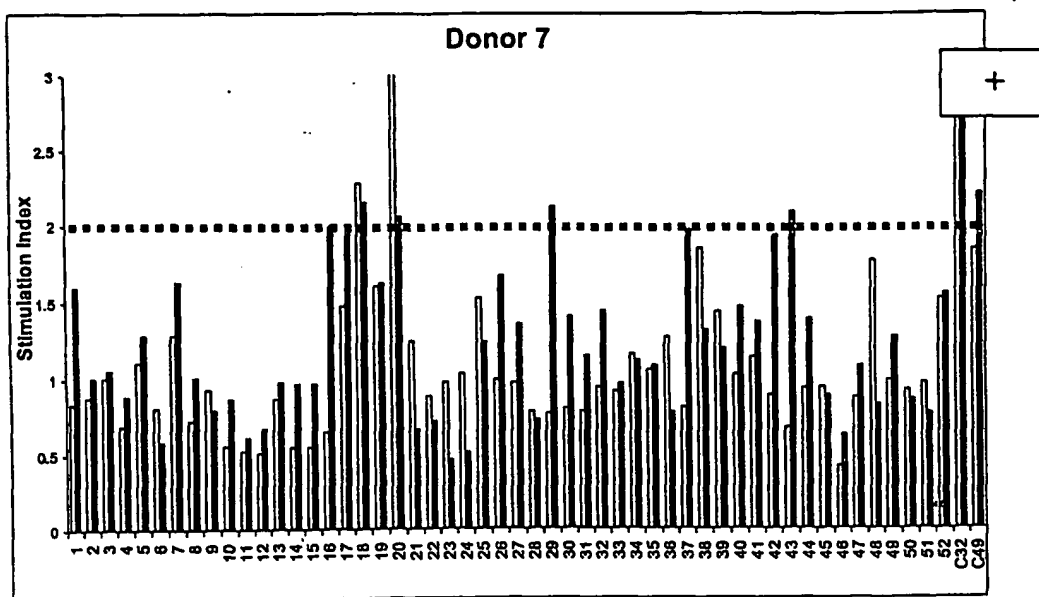


FIGURE 7f

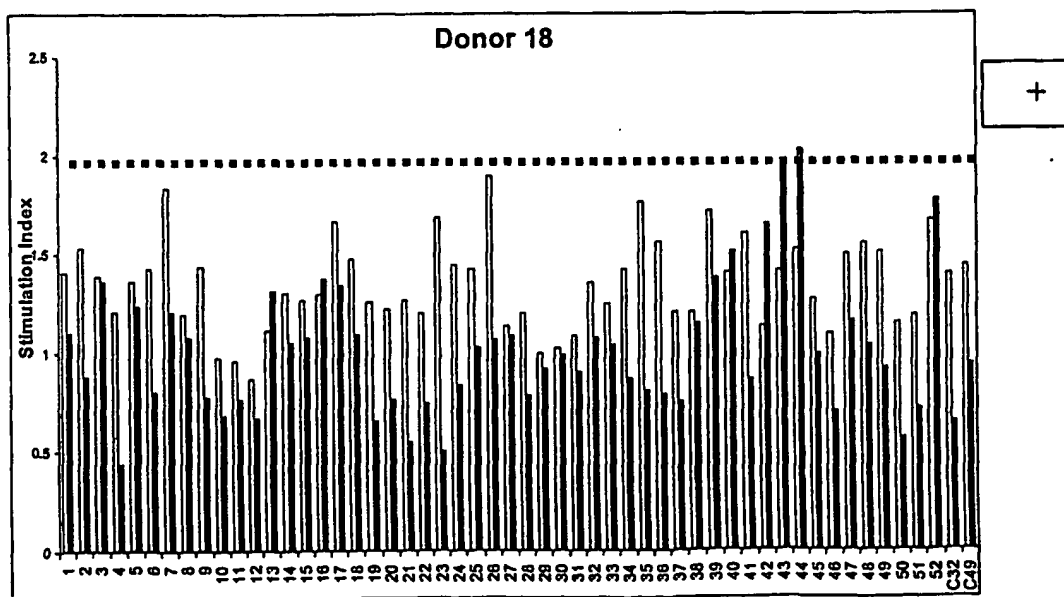


FIGURE 9

Donor #	Overlapping Epitope*			Single peptide Epitope**	
	Number of peptides	Peptide ID#	Epitope Region	Peptide ID#	Epitope Region
1	1			21	R1
1	2	43, 44	R2		
2	1			23	R1
3	1			45	R2
5	1			44	R2
7	3	17,18,20	R1		
7	1			43	R2
18	1			44	R2

FIGURE 10a

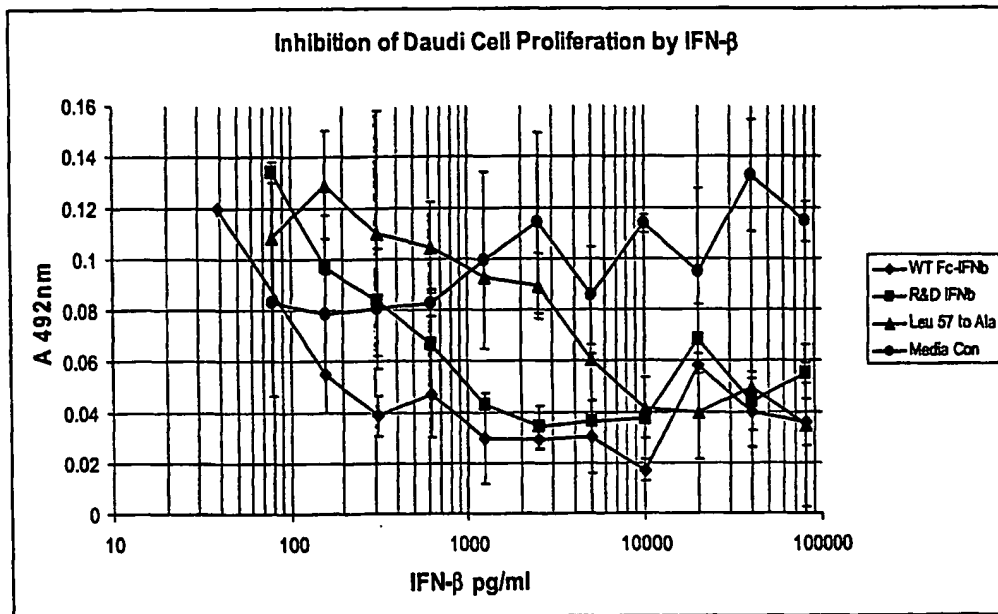


FIGURE 10b

